

MICROFLORA IN BEET SUGAR EXTRACTION



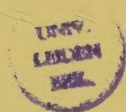
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MICROFLORA IN BEET SUGAR EXTRACTION.

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This dissertation is a summary of the following communications referred to in the text by Roman numerals. In addition to these publications some unpublished results have been included.

- I. Haskå, G., Nystrand, R. 1982.
Size and activity of the microflora in beet sugar extraction.
La Sucrierie Belge 101: 131–144.
- II. Nystrand, R., Haskå, G.
The composition and biochemical activity of the thermophilic microflora isolated from beet sugar extraction.
Accepted for publication in Zuckerindustrie.
- III. Nystrand, R.
Saccharococcus thermophilus gen. nov., sp. nov. isolated from beet sugar extraction.
System. Appl. Microbiol. 201 In press.
- IV. Nystrand, R.
Disinfectants in beet sugar extraction.
Submitted to Zuckerindustrie.

1. Introduction

The processing of sugar beets into sugar is a complex process which involves several steps where micro-organisms can cause economic problems. In the early days of the sugar industry the process was a batch process. Since about 1960 the process is operating continuously and only the last step, i.e. the crystallization, being batch-wise. At the same time the capacity of the factories has increased and thus the total material flow. If the process is not carefully monitored, micro-organisms can cause considerable losses of sucrose and other damage such as corrosion. A schematic flowchart of the process is shown in Figure 1.

The most important process step where micro-organisms can cause losses of sucrose is the extraction. This process step operates continuously in a counter-current mode and may be compared to a continuously operating fermentor. The most common and important micro-organism which has been reported to grow and dominate during the extraction step is *Bacillus stearothermophilus* (1, 12, 20, 43).

The losses of sucrose due to microbial activity are estimated to be in the range 0.05–0.5 % of the weight of the beet. In order to decrease the losses of sucrose due to microbial activity disinfectants, usually formaldehyde, are added.

A survey of the microbiology of the sugar manufacturing process, especially the conditions during the extraction (I) is presented below.

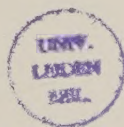
Furthermore, the size and composition of the microflora is described. In the extraction vessel the microflora and its activity have been very carefully monitored and the results will be discussed and compared to different detection methods. The effect of disinfectants on the composition of the microflora and microbial activity has been investigated. Several disinfectants and combinations of such have been tested in order to find an alternative system to formaldehyde (IV).

Many thermophilic micro-organisms isolated from the extraction step have been biochemically and morphologically characterized (II) in order to have a knowledge of the potential properties of the microflora. A Gram positive aerobic, heterotrophic, thermophilic non-spore forming coccus has been found to dominate in all the investigated factories. This organism was found during all campaigns of study. The sugar factories do only operate about three months in the autumn and this is called campaign. This organism, *Saccharococcus thermophilus* (III), belongs to a suggested new genus and species.

2. A brief technical description of the manufacturing of beet sugar.

The manufacturing of sugar from sugar beets is a complicated process. A brief description of the technical process is given here with reference to Figure 1.

The beets are delivered to the factory and stored for a short time before they are flumed (i.e. flushed by water and transported via a system of narrow trenches) to the beet washing. In this step the beets are washed by water in a rotating machinery thus removing most of the adhered soil. The amount of soil delivered with the beet is normally 15–30 % of the weight of the beet.



Just before entering the extraction vessel, the beets are sliced into cossettes (i.e. V-shaped shreds) by rotating knives. In the extraction vessel, which has a volume of 150–500 m³, the cossettes are treated with a counter-current flow of water heated to 70°C. The plant cells are hereby killed and the sucrose is extracted. The product from the extraction is called raw juice and normally contains about 15 % sucrose. The extracted cossettes are then pressed and the water formed is returned to the extraction vessel.

In addition to sucrose the raw juice contains many organic and inorganic substances which must be removed. By the addition of slaked lime organic substances are precipitated. This is done in the preliming and the mainliming steps. Most of the invert sugars, glucose and fructose, are destroyed during the liming.

The pH increases from 6.2 to 12.5. Carbon dioxide is then blown into the juice forming calcium carbonate. This is called the first carbonatation. Most of the precipitated impurities are adsorbed on the calcium carbonate and thus removed in the following filtration or sedimentation. In a second carbonatation is the amount of calcium ions reduced to a minimum.

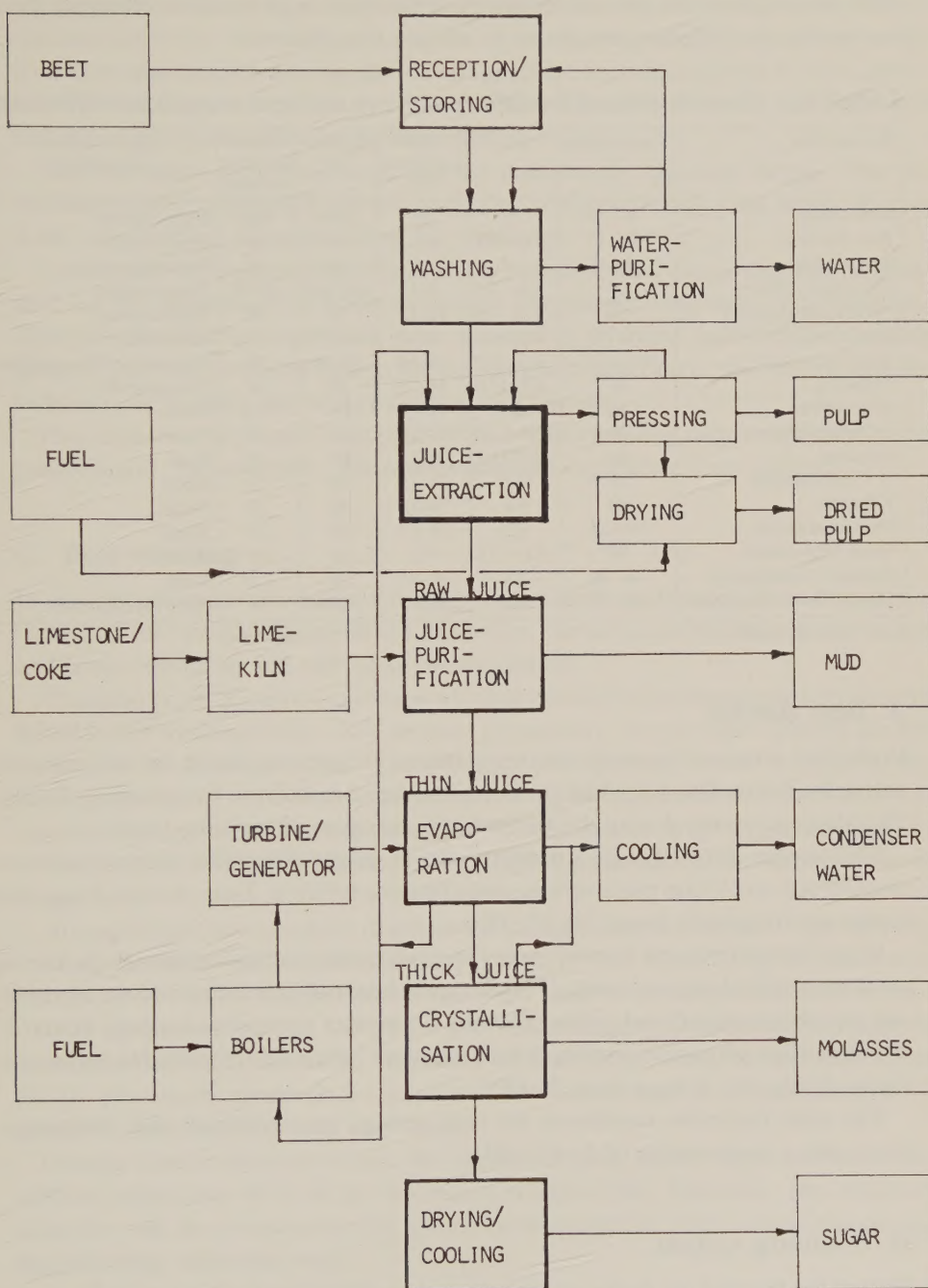
The juice is now known as thin juice and most of the non-sucrose substances have been removed. The next process is a five step evaporation station during which the sucrose content increases from 14–15 % to 65 %. The obtained juice is called thick juice.

The thick juice is then boiled batchwise under vacuum to avoid excessive temperatures. The boiling step aims to give about 50 % of the sucrose as crystals and the rest of syrup. The product of the boiling is known as magma and is cooled to 40–50°C. The sucrose crystals are removed from the magma by centrifugation. The syrup is mixed in the thick juice and sent to further crystallisation steps. Finally, the crystallized sucrose is dried by hot air and stored in large silos before it is packed and transported to the consumers.

For a detailed description of the sugar manufacturing process the book “Beet Sugar Technology” (McGinnis, 3rd ed. 1982) is recommended.

FIGURE 1.

Schematic plan of the beet sugar manufacturing.



3. The microbiology of the different process steps

This section describes the microbiology of the beet sugar process. Relevant parameters for the different process steps are given in Table 1.

TABLE 1. *Characteristics of the different steps in the sugar manufacturing process.*

Process step	Temperature °C	pH	Dry substance %	Volume m ³	Retention time minutes	Type of active microflora
Beet storage	0- 15	7.5	25	12000	3000	Psychrophilic
Beet fluming	0- 15	7.5- 9.0	n. a.	40	10	Psychrophilic
Beet washing	0- 15	7.0	n. a.	80	20	Psychrophilic
Extraction system	70	6.0- 6.5	0-15	150-500	30-90	Thermophilic
Press water system	70	5.0- 6.5	14-15	25	30	Thermophilic
Raw juice system	35- 40	6.0- 6.5	14-15	10	2	Mesophilic
Raw juice cistern	25 or 55	6.0- 6.5	14-15	40-80	20	Mesophilic/Thermophilic
Preliming	60	6.0-11.0	14-15	80	30	Thermophilic
Mainliming	80	12.5	14-15	65	20	None
I. Carbonatation	85	10.8-11.2	14-15	45	10	None
Filtration	80	10.8-11.2	14-15	85	20	None
II. Carbonatation	95	9.2	14-15	40	10	None
Filtration	90	9.2	14-15	40	10	None
Thin juice system	70-128	9.2	14-15	80	20	None
Thick juice system	70	8.6- 8.8	70	60	60	None
Boiling/Crystallization	70- 80	8.8	70-92	140	150	None
Crystal/Syrup separation	40- 50	7.2	99/90	100	30	None (Mesophilic)

n. a. = not applicable

A. Beet storage

In the first process step many micro-organisms are present due to the soil contaminating the beets. The microbial growth is supported by sucrose from damaged beets. This damage occurs during the harvest and transportation of the beets.

The temperature (0-15°C) permits growth of psychrophilic micro-organisms, *Bacillus* spp and Gram positive non-spore forming rods e.g. *Leuconostoc*. Fungi and yeasts are frequently found (8, 37, 73).

When frozen beets are thawed, many bacteria produce slime capsules from sucrose consisting of dextrane or levane (74). Since the beet cells are killed and the cytoplasmic membrane destroyed during thawing no barrier to sucrose leakage exists. In addition, the endogenous metabolism of the sugar beet is also responsible for sucrose losses during the storage time (9, 64).

The most favorable conditions for beet storage requires fresh and undamaged beets and a temperature of 2-4°C (83).

B. Fluming system

During the fluming the beets are transported by water into the beet washing. In this step some sucrose is extracted from damaged beets. The most common organisms found are *Pseudomonas* spp, *Flavobacterium* spp and Gram positive, non-spore forming rods. Due to the low temperature (0-15°C) mainly psychrophilic organisms are active (72). Mesophiles can be active, but the temperature is in the lower range.

Thermophilic species, especially *Bacillus* spp may be isolated, but they are probably not metabolically active in this system. The total cell count is high, 10^7 – 10^8 cells/ml and the number of colony forming units (CFU) at 8°C is 10^4 – 10^6 /ml.

Corrosion is caused by acids produced from sucrose by micro-organisms. Lactic acid and all lower fatty acids are present in the fluming water. In addition to the corrosion unpleasant odor may also develop, if the microbial activity is not suppressed. The activity is controlled by adding sodium hypochlorite to the fluming water and the pH may be increased by the addition of lime.

The microflora is heterogenous and the dominating organism change. The environment during fluming is not as constant as during extraction. One reason for this is the temperature decrease during the campaign.

In this study different dominating bacteria were recorded, but no organism dominated for the duration of a whole campaign or between campaigns. Furthermore, different dominating organisms were isolated in different parts of the fluming system. This was probably due to the technical construction of the system and the technique of fluming the beets from the beet storage.

Pseudomonas spp and Gram positive non-spore forming rods were found to be predominant. Occasionally, *Flavobacterium*-like organisms were isolated.

C. Beet washing

In this step the beets are washed in water under rotation. Most of the soil upon the surface of the beets is thus removed. However, the surface of the beet is very rough and some remaining soil will enter the extraction.

The same types of micro-organisms which are found in the fluming system are also found in the washing water. The physical parameters, temperature and pH are the same as in the fluming system. The microbial problems are similar to those of the fluming system, i.e. corrosion and bad odor. In addition, an aerosol is formed which represents a hygiene problem as the micro-organisms in the droplets may be inhaled.

The number of micro-organisms in the washing water and in the fluming water is comparable.

Attempts have been made to diminish the microflora on the surface of the beets by treating the beets with lime solution at pH > 12 (71) or by spraying the beets with disinfectants. Calcium hypochlorite has been used in a concentration of 0.008–0.01 % on beets (60). An iodophore (36) and Chlorazin 90 (chloroderivative of isocyanuric acid) (22) have also been used for beet disinfection. In this study Fekta RT (a quaternary ammonium compound) has been tested in a concentration of 0.0025 %, but the effect was very small.

Usually these treatments reduce the number of living micro-organisms and some authors report that 99 % of the microflora is killed (36). However, any obtained reduction will be counteracted by growth of thermophilic micro-organisms during the following extraction step.

D. Extraction

Immediately before entering the extraction the beets are cut into cosettes by the slicing machines. The material bulk flow in the extraction is large (1). The cosettes

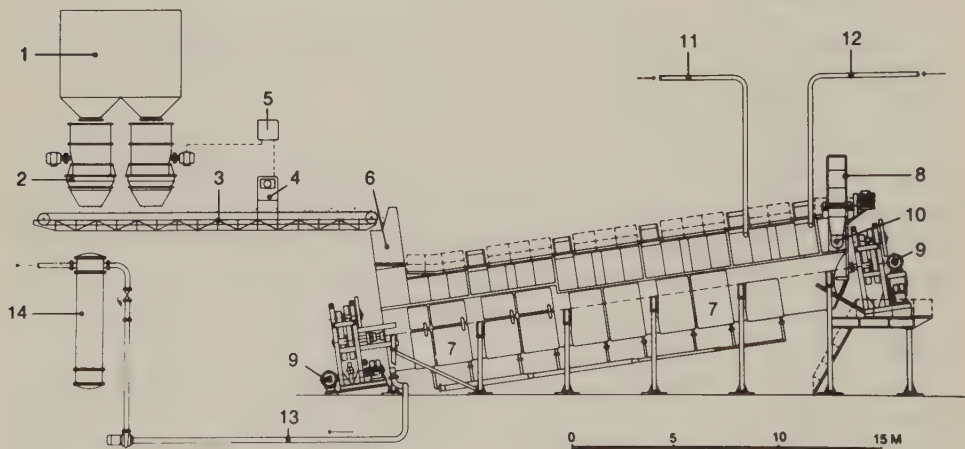
are treated with 70°C water in a counter-current mode to extract the sucrose. In the extraction step the conditions are quite different from those of the previous steps in the process. The temperature is raised from 0–15 to 70°C which means that instead of the earlier dominating psychrophilic and mesophilic organisms, thermophilic organisms now will dominate (1, 12, 20, 27, 42, 44, 45, 58).

The cells of the beets are killed and the sucrose is extracted. The sucrose concentration in the extraction vessel forms a gradient from almost 0 % in the water end of the vessel to approximately 15 % in the raw juice end. The extraction vessel is a nutritionally rich environment for the thermophilic bacteria. As a consequence of this the extraction is the most sensitive process step with respect to microbial activity.

The microbiology of the extraction will be described in more detail in section 4.

The extraction can technically be performed in different ways. In Sweden two different systems are used: (1): De Danske Sukkerfabrikker (DDS) extraction trough and (2): Buckau and BMA extraction towers (Fig. 2–3). In the tower extraction system a small scalding trough is used to heat the cossettes before entering the extraction tower. The microbiological and physical conditions in the scalding trough are the same as in the DDS extraction trough.

FIGURE 2.

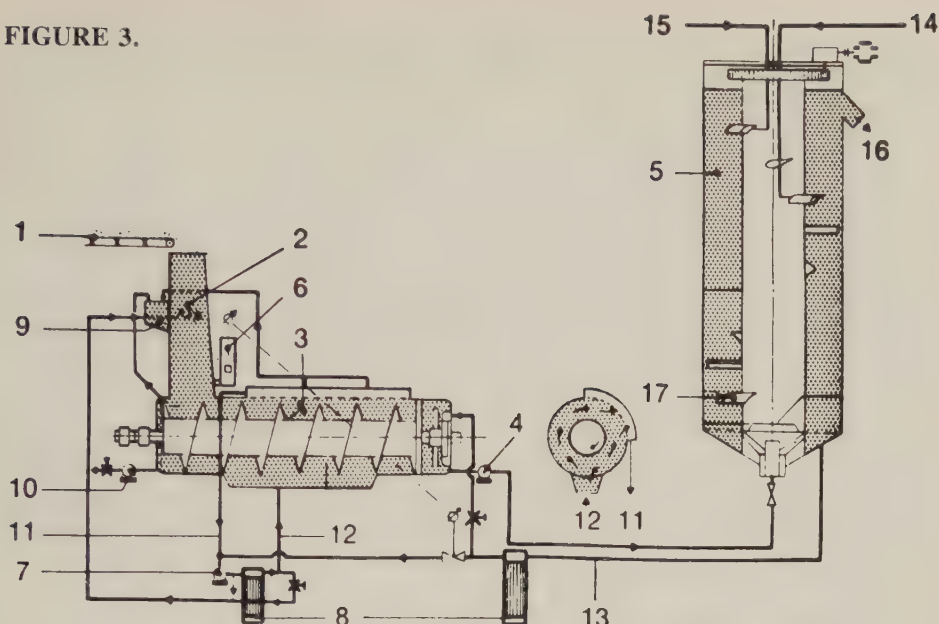


A "De Danske Sukkerfabrikker" (DDS) extraction trough.

- | | |
|-------------------------------------|--------------------------------------|
| 1 =beet bin | 8=blade wheel for removing beet pulp |
| 2=slicing machines | 9=motors and gears for helixes |
| 3=cossettes conveyor belt | 10=beet pulp worm to presses |
| 4=conveyor weighing machine | 11=press water |
| 5=regulator for amount of cossettes | 12=water |
| 6=entrance shaft for cossettes | 13=raw juice |
| 7=rotating iron helixes | 14=pre-heater |

(From "Svenskt socker" Ed. Laudon, A. CWK Gleerup 1970)

FIGURE 3.



Buckau extraction tower with scalding trough.

1=cossettes conveyor belt

2=entrance shaft for cossettes

3=scalding trough

4=cossettes pump

5=extraction tower

6=juice level regulator

7=cross-flow pump

8=pre-heater

9=anti foam

10=raw juice pump

11=suction pipe to pump

12=pressure pipe from pump

13=tower juice pipe

14=press water

15=water

16=beet pulp outlet

17=agitator

(From "Svenskt socker" Ed. Laudon, A. CWK Gleerup 1970)

E. Press water system

When the extracted cossettes have left the extraction vessel they are treated in a system of presses. The press water formed is pumped back into the extraction vessel. In the press water system the temperature is about the same as during the extraction. The microflora has the same composition as during the extraction, but the number may differ. The same organism dominated during extraction and in the press water (I, III).

Thus, the press water system can, from a microbiological viewpoint, be regarded as a part of the extraction system. It is essential that this part is kept at a high temperature and disinfected like the extraction. If not, the press water system can act as an inoculation source to the extraction.

In some sugar factories the press water is heated to 95°C in a heat exchanger in order to inactivate the microflora.

The buffer capacity of the press water is low compared to the extraction juice. This means that a drop in pH due to the formation of acids can first be detected in the

press water. Just before the press water is returned to the extraction vessel it is generally acidified to pH 5 by the addition of sulphuric acid.

F. Raw juice system

The raw juice is the "product" of the extraction. The temperature is 35–40°C. The low temperature is achieved by heat exchange when the extraction juice heats up the incoming cossettes. The cossettes are washed by the extraction juice which means that soil adhered to the cossettes comes more or less directly into the raw juice.

The microflora of the raw juice is predominately mesophilic. Most thermophilic organisms isolated originated from the extraction. The number of mesophilic bacteria is in the range of 10^6 – 10^7 CFU/ml. Due to the very rapid flow of the raw juice and the small volume of the piping there is no growth of any single dominating species. A large variety of mesophilic bacteria can be isolated from the raw juice e.g. *Bacillus*, *Pseudomonas*, *Lactobacillus*, *Flavobacterium*, *Clostridium* and *Corynebacterium*. Fungi and yeasts are also present, but their numbers are small (79). Species of all these genera have been isolated in this study.

G. Raw juice cistern

In some sugar factories a raw juice cistern is to be found. This acts as a buffer for further processing i.e. gives an even flow in the process. In Örtöfta sugar factory the temperature of the raw juice is held at 55°C.

Throughout this study the microflora was found to be constant. The dominating organism was a Gram positive, non-spore forming, facultatively anaerobic rod. The organism was characterized as a facultatively thermophilic *Lactobacillus* sp. The number was 10^4 – 10^6 CFU/ml. It was not detected in the extraction vessel. The raw juice cistern can also be held at a lower temperature, 25–30°C (Karpalund). In this case the microflora consists of mesophilic bacteria. The dominating species was also characterized as a *Lactobacillus* sp but this organism was mesophilic and could not grow at 55°C. The number was 10^4 – 10^6 CFU/ml. In this cistern the microflora was more varied compared to the microflora in the 55°C cistern.

The raw juice cisterns are disinfected by the addition of formaldehyde but to lower concentrations than during extraction. The frequency of addition is also lower. This is a reason why the dominating species is stable.

The main metabolites produced by the dominating species in the raw juice cisterns were acetic acid and L-lactic acid.

Interestingly, the dominating thermophilic species from the extraction could not establish themselves in the 55°C cistern.

H. Preliming

In the preliming the temperature is approximately 60°C. Lime is added in order to remove non-sugar material from the juice. The pH is increased from approximately 6.2 to 11. In the first three compartments a Gram positive non-spore forming facultative anaerobic rod was found. The number was highest in the first compartment, 10^6 CFU/ml, progressively decreased to zero in the fourth compartment.

The dominating organism was related but not identical to that found in the raw juice cistern. The main metabolite was L-lactic acid in this case.

Desinfection can be carried out by the addition of formaldehyde when necessary. Investigations of the sucrose losses in the preliming juice have been performed by Hollaus and Wieninger (32) and Pollach and Wieninger (67). They found that micro-organisms were able to produce L-lactic acid and that some isolates were invertase producers i.e. *Bacillus licheniformis* and thus caused sucrose losses.

I. Mainliming

In this process step the pH is 11–12.5 which is too high to permit growth. In addition, the temperature is almost 80°C. No active growth could be shown and organisms found were sporeformers.

J. Thin juice system

The clear juice obtained after liming, carbonatation and filtering is known as thin juice and normally contains 14–15 % sucrose. The juice temperature is 60–80°C. No growth was detected during this part of the process. A few spore formers could be isolated, but their number was low.

However, in factories where ion exchanges are used the temperature is lower (28) and growth may occur.

K. Thick juice system

The thin juice is heated in a series of evaporators under pressure to 133°C and at 2.5 bars in the first and to 70°C and at 0.3 bar in the final evaporator respectively. The thick juice produced contains approximately 65 % sucrose and has a temperature above 70°C. It is further processed by batchwise boiling under vacuum into a mixture of crystals and syrup.

No micro-organisms grow during this part of the process. As in the case spore formers can be isolated, but they are inactive in the system.

4. The extraction

The above mentioned conditions in the sugar processing indicate that it is during the extraction that micro-organisms cause considerable problems i.e. losses of sucrose and formation of acids.

The extraction vessel is a thermophilic environment which is characterized by the following: a) high temperature (70°C), b) a retention time of 20–30 minutes for the extracting agent and about 60 minutes for the cossettes, c) a pH of 6.2 ± 0.2 , d) a redox potential of +50 to –300 mV in extraction troughs and below –300 mV in extraction towers, e) a high amount of sucrose and finally, f) the disinfectant used. The size of the material flow in the extraction is shown in Paper I.

The total number of micro-organisms found is large, approximately $1-2 \times 10^7$ cells/ml (I), which is in the same range, $1 \times 10^6 - 1 \times 10^8$ cells/ml, as other researchers have reported (16, 85). Many different organisms were isolated from the extraction vessel (II). The spore forming Gram positive rods constituted 35 % of the isolates, but they were never found to dominate during the extraction. Klaushofer and

Hollauss (43) and Abdou (1) described only spore forming thermophilic organisms in their investigations and they considered this group of bacteria to be the most important one. The organisms were members of the *B. stearothermophilus* group. There is little information in research literature concerning other dominating organisms during the extraction.

Since about 1955 most authors have pointed out that the most important micro-organisms present in the extraction are members of the *Bacillus stearothermophilus* group (1, 12, 20, 43). This is especially valid for trough extractions but also for tower extractions. Furthermore, species of the genus *Clostridium* (31, 42) are found in tower extractions, which are more anaerobic compared to trough extractions.

In contrast, the dominating organisms found in the Swedish extraction systems are Gram positive aerobic thermophilic cocci (III). They have been isolated from all Swedish sugar factories. The coccus grows in all parts of the extraction vessel when formaldehyde is used as the disinfectant where the redox potential exceeds -300 mV. This coccus is suggested to be a new genus and species tentatively named *Saccharococcus thermophilus*. Another type of bacterium was found when the disinfectant used was hydrogen peroxide instead of formaldehyde. This organism was a Gram positive non-spore forming rod characterized as a thermophilic *Lactobacillus* sp (IV).

It is interesting to note that in spite of the large number of different organisms present in the extraction vessel only a few have been reported to be of any economic importance i.e. losses of sucrose (27, 69). The composition of the microflora and especially the dominating species was found to be stable during different campaigns. Minor disturbances of technical nature or even complete stops, including emptying of the extraction vessels for 24 hours, did not change the microflora.

When the disinfectant was changed from formaldehyde to hydrogen peroxide the dominating species also changed (IV). Only such changes of the shock chemical (i.e. a disinfectant which is added in large quantity in a very short time) resulted in a change of the microflora. The addition of dithiocarbamates, continuously or periodically, did not lead to any change probably due the low concentrations used. Thus, only considerable changes in the environmental conditions result in a new dominating organism.

The microbial activity during the extraction is of great interest since it represents losses of sucrose.

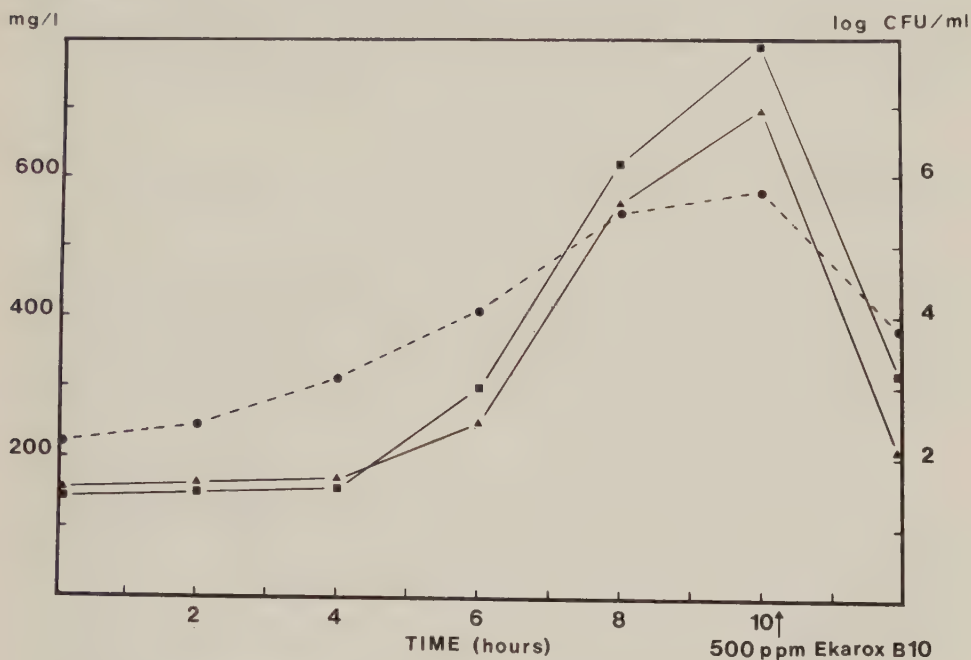
L-lactic acid has been found to be the most common metabolite formed by the microflora both during the extraction (I) and in laboratory studies of isolates (II, III, IV). However, acetic acid and ethanol were also found. Similar results were obtained in an investigation of the biochemical transformations in extraction juices by Winström-Olsen et al (87) in Denmark.

Klaushofer et al (42, 45) studied the ability of isolates to produce acid from sugars. They found that L-lactic acid was the most common acid formed. Lorenz (52) and Shore (75) stated in their investigations that L-lactic acid constituted 75–95 % of the total amount of acids formed. The formation of invertase by micro-organisms also causes sucrose losses. However, in this study rather few isolates were shown to produce invertase. The frequency of invertase producers was 3–11 % for most groups of bacterial isolates, but was 18 % for the group of 55°C Gram positive cocci (II). The ability to form invertase has been studied by many authors (32, 46, 47, 64,

86). The organisms found to be invertase producers could generally also produce acids e.g. L-lactic acid from glucose and fructose.

It is not only micro-organisms which are responsible for the invert sugar formation during the extraction. The sugar beet itself contains invertase of at least two different types (9).

FIGURE 4.



Growth of *Saccharococcus thermophilus* (an invertase producing strain) (● — — ●) and formation of invert sugars, glucose (■ — ■) and fructose (▲ — ▲) in the DDS trough extraction at Örtofta sugar factory. Temperature 68°C. The addition of 500 ppm Ekarox B 10 is indicated by an arrow.

The amount of invert sugars present in the extraction was usually 100–300 mg/l of both glucose and fructose in this investigation. When the dominating bacterium was an invertase producer, the amounts of glucose and fructose increased. An example of this is shown in Figure 4. When the microflora was inactivated by a disinfectant, the amounts of glucose and fructose decreased rapidly. The concentrations of these two sugars were comparable. In the work of Winström-Olsen et al (86) the amount of fructose was low and constant while the amount of glucose varied. When disinfectant was added, the amount of glucose increased while the amount of fructose was unchanged. The authors concluded that most of the L-lactic acid formed was produced from glucose and not from sucrose. However, such a pattern was not evident in this work.

The temperature is a very important parameter during the extraction. The best method to prevent microbial activity is to keep a very high temperature, at least 75–80°C. The number of species which are able to grow at such high temperatures are few.

Unfortunately this is not possible for practical reasons. Pectine and other substances are extracted from the beet and will cause clogging of filters later in the process. The temperature used must therefore represent a compromise. The highest possible temperature with fresh and healthy beets is 72–74°C and normally 70° is used. For technical reasons or if the beets are of bad quality temperatures down to 65°C are sometimes used. These low temperatures always result in significant microbial sucrose degradation. The losses of sucrose during the extraction are in the range of 0.05–0.5 % of the weight of the beet (19, 38). A decrease in pH by 0.5 units and a *Bacillus* sp as the dominating organism will give a sucrose loss of 0.16–0.19 % and for a *Clostridium* sp the corresponding figure will be 0.10–0.13 % (28).

5. Detection methods

In the sugar industry there is a need of reliable methods for detection of micro-organisms and their activity. During the extraction it is especially important to detect growth of microorganisms as early as possible, in order to prevent losses of sucrose.

The microbial activity is carefully monitored using several detection methods. The results of these measurements are used to determine the disinfection programme and the effect of the disinfection.

A broad variety of detection methods are available for determination of micro-organisms and their acitivity. Cultivation methods and microscopic examinations are used to detect the presence and kind of micro-organisms. Physical and biochemical methods are further used to determine the microbial activity. The methods used are listed in Table 2.

In this section a review of the different methods is presented and the methods are compared to the results obtained in this investigation.

TABLE 2. *Detection methods.*

Group of methods	Method	Time	Type of method and where to be performed
Cultivation	Agar surface plate	> 24 hours	A
	Agar pour plate	> 24 hours	A
	Membrane filter cultivation	> 24 hours	A
	Micro slide culture	4–6 hours	A
Microscopic	Phase contrast, direct observation	5–10 minutes	B
	Congo red staining	20 minutes	B
	Membrane filter, stained	30 minutes	B
	Membrane filter, fluorescence	30 minutes	B
	Stained antibody technique	30 minutes	B
	Nitrite	15 seconds	C
Biochemical	Hydrogen sulphide	30 seconds	C
	Lactic acid	20 minutes	B
	Gas chromatography	30 minutes	A
	Redox indicators	0.5–2 hours	B
	Adenosine triphosphate	20 minutes	A
	pH	continuous	C
Physical	Redox potential	continuous	B
	Temperature	continuous	C

A = Research method. Research laboratory.

B = Routine method. Factory laboratory.

C = Routine method. Extraction operator.

A. Cultivation methods

The cultivation methods give information on different types of bacteria present in the samples and the number of CFU/ml.

These methods are generally valuable in basic research work and in special investigations. They are not suitable as methods for routine monitoring during the extraction. However, they may be used to determine the dominating species. The time of incubation required is at least four hours for the slide micro method (3, 28) and 1–2 days or even more for other cultivation methods.

The composition of the cultivation substrates used is important. A suitable substrate should give as many different organisms as possible and in the highest possible number (I).

The substrates used in this investigation allowed all dominating organisms to grow very well. This was controlled by microscopic examination of the samples. In no case a large amount of cells was observed without corresponding growth on the media used.

In this study the agar substrates were also used for isolation of organisms, which were used in special investigation i.e. biochemical properties (II), taxonomy (III) and susceptibility to disinfectants (IV). The selection of isolates was based on colony morphology. The agar substrates were examined and the colonies were selected according to the following:

1. Dominating organisms.
2. Frequent organisms, but not dominating.
3. Organisms which were present and recognized for a long period of time but in low numbers.

The dominating organisms constituted 99 % or more of the CFU number. Other frequent organisms constituted 1–10 % of the CFU number. They were recorded in their highest number when no dominant organism was present. The third group was easiest to isolate when the dominating organism was suppressed or not present.

The organisms were isolated from the routine samples at three different temperatures 35, 55 and 70°C.

The use of this principle of isolating organisms resulted in that almost all 70°C organisms were isolated. Compared to the other temperature groups in the routine samples, there were a lower number of different organisms in the 70°C group. In the 55°C group the organisms isolated were in the third group, except for a few organisms which were in the second group.

The 35°C organisms isolated were all in the third group.

B. Microscopic methods

Microscopic methods are rapid and simple to perform in the laboratory, although they require some experience from the personnel. The methods give information of the number of cells present in the samples and some information of the morphology and the cell arrangements.

The microscopic methods can be divided into the following:

a. **Direct observation of a sample without any pretreatment.**

In this method a phase contrast or interference phase contrast microscope should be used. The samples can be concentrated by centrifugation. It is also possible to count the cells in a counter chamber.

In this investigation phase contrast microscopy was used routinely to confirm the presence or absence of high numbers of cells especially of dominating organisms. It was very easy to establish the presence of the Gram positive coccus *Saccharococcus thermophilus* using this method. As the coccus occurs in clusters (III), it was simple to observe. Furthermore, the size and the number of cells in the clusters could be estimated (III).

b. **Staining methods.**

Conventional staining methods can be either positive or negative. The positive staining methods such as methylene blue and erythrosin can be performed directly on a dried sample or on a membrane filtered sample.

The negative staining methods e.g. nigrosin and Congo red (85) must be mixed with the sample. Due to the large amount of particles present in the samples it is difficult to distinguish cells from other particles in this method compared to the other microscopic methods.

c. **Staining with different fluorescent dyes on membrane filtered samples.**

The stains used in this study were acridine orange (AO) and fluorescein diacetate (FDA) (I). The AO method gives the total number of cells while the FDA method only stains cells which can hydrolyze the FDA to form free fluorescein. These methods are rapid and simple to perform. The methods require, however, a UV-microscope.

The cells stained with FDA can be regarded as potentially active organisms. All the dominating species stained very well with FDA in this study.

A special variety of fluorescent staining was used by Klaushofer et al (41). They produced stained antibodies which attached to cells of *Bacillus stearothermophilus*. In this way it was possible to identify and enumerate this bacterium.

C. Nitrite test

The nitrite test is widely used (13, 39). This test indicates the presence of micro-organisms which can reduce nitrate to nitrite. The test is very simple to perform using nitrite sticks (57). The analysis may also be performed by spectrophotometric measurement (18).

The advantage of the test is that it is rapid and simple. It can be performed by untrained personnel.

However, not all dominating organisms can reduce nitrate to nitrite and these organisms will not be detected with this test (III). This is also the case with organisms which can rapidly reduce the nitrite further to nitrogen gas.

In this investigation 23–51 % of the isolates in different groups of bacteria or totally about $\frac{1}{3}$ of the thermophilic isolates could reduce nitrate to nitrite (II). Thus, these results indicate that the nitrite test by itself is not a reliable test method for detecting microbial activity.

D. Hydrogen sulphide

The ability of some micro-organisms to produce hydrogen sulphide can be used as a detection method especially in tower extractions (29). The test requires that sulphur in utilizable form is present. The anaerobes isolated from the Swedish extractions were not able to produce detectable amounts of hydrogen sulphide at 70°C but some 55°C isolates were (II).

Among the aerobic and facultative anaerobic isolates a small number of the 55°C isolates produced hydrogen sulphide, but none of the 70°C isolates.

This method was not considered to be useful in the Swedish extractions since no sulphur dioxide is added here.

E. Lactic acid

Measuring the amounts of lactic acid, especially L-lactic acid is a frequently used method for the detection of microbial activity (49, 56, 65, 70).

As mentioned above, L-lactic acid is the most common metabolite formed in the extraction (I, II).

For this reason L-lactic acid is a suitable metabolite to analyse. The test is performed using an enzymatic test kit which, with a slight modification, can also be used to determine D-lactic acid (49).

In this investigation the following assessments were used concerning the amount of L-lactic acid:

0– 50 mg/l: Very good. No microbial problem.

51–100 mg/l: Good. Slight microbial activity.

101–200 mg/l: Indication of growth of a dominating species.

201–500 mg/l: Problem. The activity should be suppressed.

> 500 mg/l: Severe problem. The activity must be suppressed.

The highest values found in this study were about 1200 mg/l of L-lactic acid. This concentration did not inhibit the dominating species. D-lactic acid was found in very small amounts, about 10 mg/l in the extraction and at only one occasion more D-lactic acid than L-lactic acid was found. The amount was 50 mg/l of D-lactic acid. None of the dominating species isolated could produce D-lactic acid.

A disadvantage in the use of the enzymatic method is that it is normally only performed 1–3 times a day, i.e., the frequency is low. However, there are automated analysis systems available (15). The analysis can also be performed by gas chromatography (I). Further on, L-lactic acid is not the only metabolite formed, although it is generally the most common one. This means that the calculated sucrose losses due to L-lactic acid formation must be considered as minimum losses.

In aerobic parts of the extraction, especially in trough extractions, other metabolites from sucrose degradation e.g. water and CO₂ occur (61). Thus, the result from a L-lactic acid analysis can be good, but there is still an ongoing growth of a dominating species (40). Pollach (67) calculated that in anaerobic systems the mol ratio formed L-lactic acid to sucrose was 1: 1 but it was only 0.04: 1 in aerobic systems.

For these reasons the samples for L-lactic acid analysis in this investigation were checked under the microscope in order to determine the presence or absence of large

numbers of cells of dominating species. No situation was, however, recorded showing no L-lactic acid accompanied by a high cell concentration or vice versa. Both the dominating species, *Saccharococcus thermophilus* and the *Lactobacillus* sp produced L-lactic acid as their main metabolite (III, IV). However, it is not certain that the conditions for L-lactic acid production were optimal, but the amounts of L-lactic acid formed were in any case large enough to correspond to high numbers of cells.

F. Gas chromatography

Gas chromatographic analysis is used for a broad spectrum of metabolites (I, II). By using an instrument with a flame ionisation detector and a suitable column packing material e.g. Chromosorb 101, lower fatty acids, alcohols, aldehydes and ketones can be detected. Generally, gas chromatography is widely used as a method of determining microbial activity and is also used in the identification of micro-organisms, especially anaerobes (4, 10, 11, 23, 51).

The gas chromatographic analysis is rather simple to perform in the laboratory but requires a trained technician. Information on identity and amounts of metabolites formed as well as type of dominating organisms may be obtained.

Compared to the other methods described this method is more advanced but it is also the method which gives most information. Modern gas chromatographs can operate automatically and more or less continuously to give information on microbial activity.

In this study gas chromatography has been used to analyse samples from different process steps as well as for the characterization of the isolates.

G. Chemical redox indicators

Chemical redox indicators are used to indicate microbial activity. The most common redox indicator is resazurin (6, 29, 59). The test is very simple to perform and is used as a routine control method. The incubation time required for the test is 1–2 hours which is too long. The test should not be used alone but in combination with pH measurement and L-lactic acid analysis.

Triphenyltetrazolium chloride (TTC) and methylene blue are also used as redox indicators (9, 35).

H. Adenosine triphosphate

Adenosine triphosphate (ATP) is present in all cells as an intermediate metabolite. The amount of ATP can serve as a measurement of bacterial number during the extraction. According to Baumgart (5) the amount of ATP in a *Bacillus* cell is 5.9×10^{-20} mol.

There are several practical problems when using the method. Firstly, all the ATP in the beets must be totally destroyed and removed before any analysis can be performed (61). Secondly, too many particles in the samples disturb the analysis. Thirdly, the equipment is complicated as well as the reactions. Thus, this analysis is not suitable as a routine method but may be used in research work.

I. pH.

Measurement of pH is the most common routine test (30, 66). It is a fast and simple test method for microbial activity. The acids produced originate from degradation of carbohydrates and therefore a pH-drop is a direct indication of carbohydrate degradation. The advantage of the pH measurement is that it can be frequently done or even continuously in order to give information on the microbial activity.

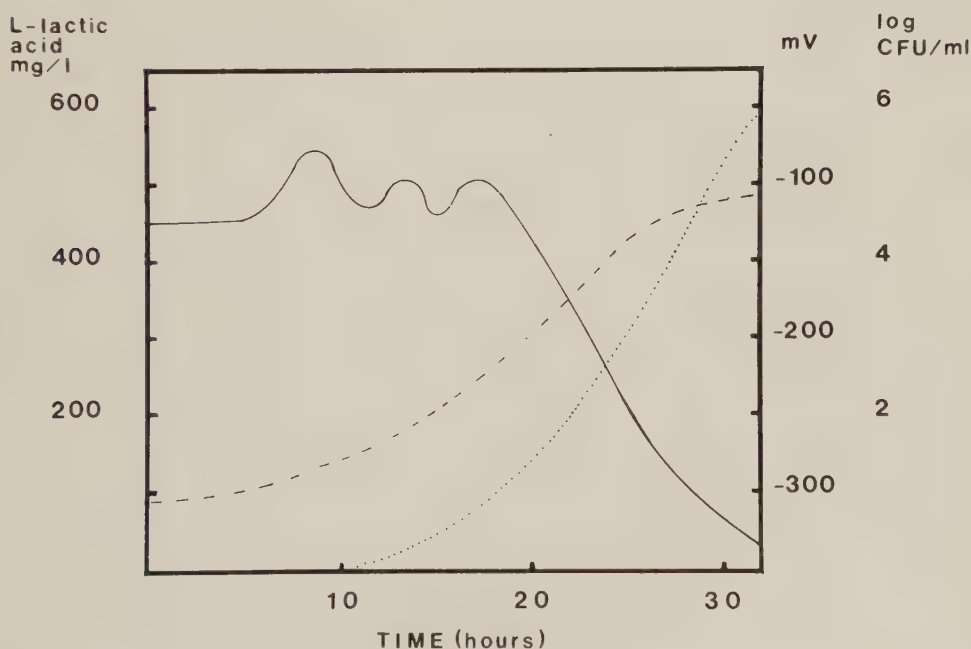
A disadvantage is, however, that an infection may already have been established when a drop in pH is recorded due for the buffer capacity of the extraction juice.

In this investigation pH was used to indicate the need to add disinfectants (IV) since it was the only test which could be routinely performed both day and night in the factory.

J. Redox potential measurement

The redox potential in the extraction vessel is influenced by the chemical reactions caused by micro-organisms. Thus, changes in the redox potential can serve as an indication of microbial activity. This method has also been investigated in the present study. First a laboratory pilot study was performed by using extraction juice incubated at 70°C. A Gram positive non-spore forming rod dominated. The main metabolite found was L-lactic acid. The changes in the redox potential were recorded and are shown in Figure 5. The electrode used was a platinum electrode

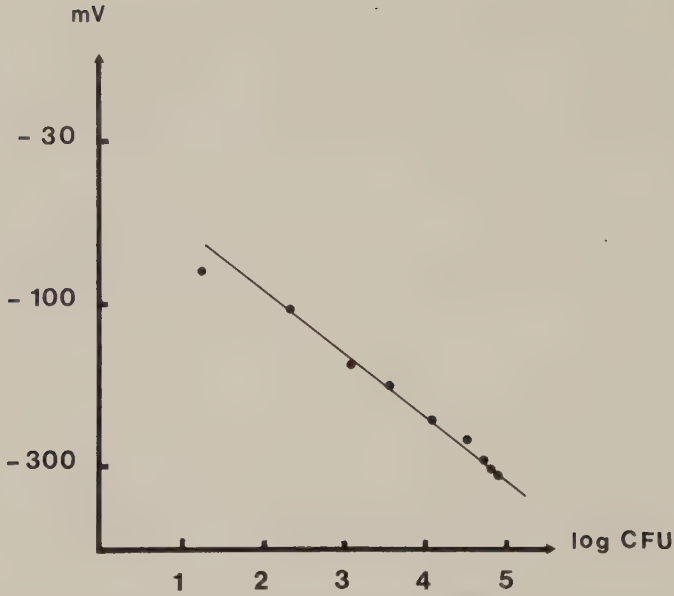
FIGURE 5.



Growth of a Gram positive non spore forming rod (*Lactobacillus* sp) in a batch culture of extraction juice (---).

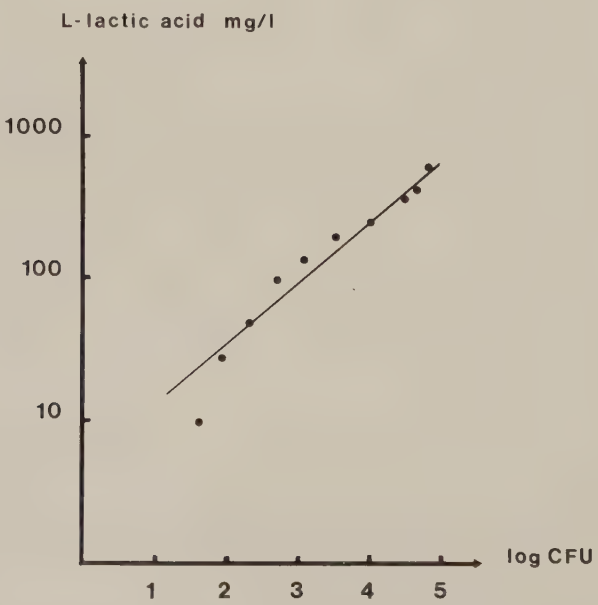
The redox potential (—) and the amount of L-lactic acid formed (.....) were recorded. The temperature was 70°C.

FIGURE 6 A



Growth of a thermophilic *Lactobacillus* sp at 70°C. A laboratory experiment (cf. Figure 5).
Correlation between redox potential (mV) (logarithmic scale) and log CFU.

FIGURE 6 B.



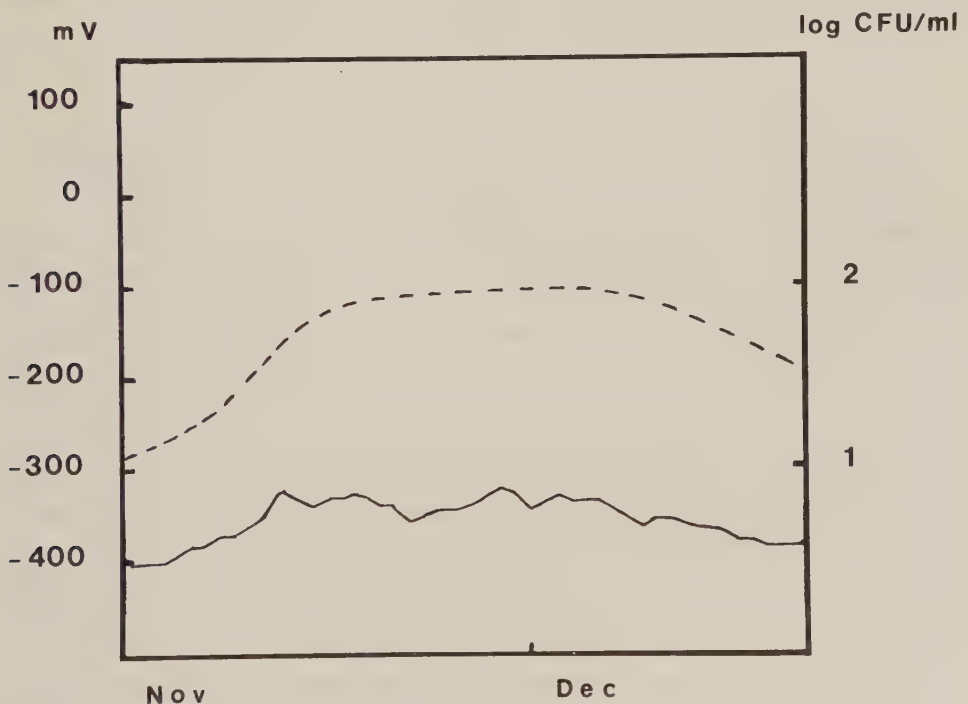
Growth of a thermophilic *Lactobacillus* sp at 70°C. A laboratory experiment (cf. Figure 5).
Correlation between the amount of L-lactic acid (mg/l) (logarithmic scale) and log CFU.

(Philips M 13) and the reference electrode was a silver/silverchloride electrode (Ingold 360-T). The signal from the electrodes was free from disturbances. The correlations between redox potential and L-lactic acid formation versus growth are shown in Figure 6 A and B. Full scale experiments were made both in an extraction trough and in an extraction tower. The platinum electrode was placed directly in the extraction vessel and the reference electrode was connected to the extraction with a salt bridge of 3 M KCl. The results obtained in the extraction trough were not acceptable. The signal varied very much and it was assumed that air was mixed in thus disturbing the system. It was possible to determine the range in which the redox potential varied in the bottom region in the extraction trough. The range was +50 to -300 mV. Due to the disturbances in the signal it was not possible to determine the changes due to microbial activity.

In the extraction tower the conditions were more constant and the obtained signal was not disturbed. The redox potential was in the range -300 to -400 mV. During the experiment period the number of micro-organisms was small and no dominating micro-organisms was present, consequently no large amounts of metabolites were produced. The results of the redox potential recordings were in agreement with these observation (Figure 7).

Nickisch and Mauch (61) investigated in a laboratory study the redox potential as an early detection method of microbial activity. They judged this method as valuable

FIGURE 7.



The redox potential in the extraction tower in Jordberga sugar factory 1979.
 Redox potential (mV): ———
 CFU/ml 70°C: - - - - -

since it is possible to detect microbial activity, e.g., formation of CO₂ which is difficult to detect with other methods.

Madsen et al (53) reported on experiments with redox potential measurements in a Danish factory with promising results.

In this investigation it was found that redox potential measurements in extraction troughs were very difficult to perform. The results were more useful in extraction towers. The method should, however, be used in combination with other methods, e.g., pH measurement and a microscopy method.

K. Temperature

Temperature measurement can be used as a detection method. If the temperature decreases, it will generally result in increased microbial activity. Therefore it is essential to know the actual temperatures in different parts of the process. If the measured values differ from the set values, corrections should be made.

The placing of the temperature sensors in the extraction vessel is critical. If the sensors are placed too close to the heating chambers, this will result in the recording of a too high temperature, which is not representative of the extraction system. In this study differences of 4–8°C were found when the temperature was measured in different parts of the extraction vessel with a calibrated mercury thermometer compared to the sensor recording system.

L. Combination of detection methods

From this review of detection methods which can be used in the beet sugar extraction it is evident that no method alone is perfect. For this reason it is not recommendable to use only one method. Combination of two or more methods gives a much better information on the situation during the extraction. Measurement of pH, in combination with L-lactic analysis is a common combination. Resazurin and measuring of pH is another combination. Measurement of pH, nitrite test and L-lactic acid is also used. In this investigation pH measurement, temperature measurement, microscopy, colony count, gas chromatography and L-lactic acid determination were used.

The presence of dominating species i.e. *Saccharococcus thermophilus* was easy to confirm as cultivation methods and phase contrast microscopy always were used in combination. This gave also the magnitude of the cell number. In addition to these methods the pattern of the gas chromatograms gave information of the microbial activity. Measurement of pH and temperature were also done as well as enzymatic determination of L-lactic acid.

Throughout this study the combination of the above mentioned methods gave unambiguous results. It was always possible to determine the type of dominating bacteria growing.

The correlation between the used methods were very good.

M. Recommendation of detection methods

Due to the fact that it in most cases is not possible to predict the species that will grow and dominate in the extraction system and their activity, it is recommended to use combinations of detection methods.

A combination of a frequent or continuous method for microbial activity i.e. pH and a method confirming if any dominant bacteria is present in high numbers, i.e. phase contrast microscopy or fluorescence microscopy with fluorescein diacetate as the staining method is recommended as routine analysis.

In order to confirm the metabolites formed, especially L-lactic acid, and the relative amounts gas chromatography or enzymatic methods can be used.

It is also recommended to get a knowledge of eventually dominating bacteria by using cultivation methods.

Action levels for the combination of detection methods must be locally determined at each factory as the conditions differs. Normally a disinfection should be carried out when passing an action level.

6. Disinfection

When a dominating organism in the extraction vessel has been detected it must be suppressed by adding a disinfectant to the extraction juice in order to prevent losses of sucrose.

There are several demands on a disinfectant which will be used in the beet sugar processing. First of all, sugar is used as a food and there must be no harmful amounts of the disinfectant in the final product. Thus, it must be possible to either remove or destroy the disinfectant in the process. The degradation products must be harmless. Secondly, the disinfectant must be effective at high temperature (i.e. 70°C) and at high concentration of organic material (i.e. the cossettes and 15 % sucrose). Thirdly, the disinfectant must not be harmful or dangerous for those who handle it.

Fourthly, the disinfectant should not be corrosive or cause damage to the equipment used in the process.

Fifthly, the costs of disinfection must be reasonable in comparison to the estimated sucrose losses.

The pH is a parameter which is important for the activity of disinfectants. In this investigation the pH in the extraction should be $6,2 \pm 0,2$. Lower pH will give hydrolysis of sucrose. The range of the pH was 5.5–6.4. Preferably, the disinfectant should be a small molecule in order to permit a better understanding of the degradation and reaction products formed.

The disinfectants in this investigation were compared to each other in a laboratory study (IV) and the most promising disinfectants were tested in full scale experiments in the sugar factories (IV).

In the extraction plants the disinfectants were added in different ways. Formaldehyde, hydrogen peroxide and Ekarox B 10 were used as shock chemicals. That is, they were added in large amounts in a short space of time. The dithiocarbamates

were added either continuously in a low concentration or periodically. The periodical addition could, for example, be an addition for two hours followed by a six hours pause. This made it possible to have a higher concentration for a short space of time compared to continuously addition.

A. Formaldehyde

The most widely used disinfectant in the beet sugar extraction is formaldehyde (I, IV). The anti-microbial properties of formaldehyde makes it suitable for use as a disinfectant (30, 78). Although formaldehyde is an effective disinfectant it has some disadvantages. It has been reported to be allergy-causing and carcinogenic. For these reasons there is an interest in testing alternative disinfectants (IV).

When formaldehyde was used as a disinfectant a Gram positive coccus, *Saccharococcus thermophilus*, dominated in all the factories investigated. The coccus had a high tolerance to formaldehyde compared to the other disinfectants tested (III). It was not possible by disinfection to free the extraction vessel from this coccus, but the number of cells and the biochemical activity decreased. By increasing the temperature above 70°C the activity of the coccus decreased and less amounts of formaldehyde were required to achieve acceptable conditions during the extraction.

B. Hydrogen peroxide

Hydrogen peroxide is also a suitable compound for disinfection in beet sugar extraction since the degradation products are harmless. Recently hydrogen peroxide has been tested in Denmark (53, 86) with good results.

In this study hydrogen peroxide has been tested and compared to other disinfectants (IV). Hydrogen peroxide was used as a shock chemical. The change of disinfectant from formaldehyde to hydrogen peroxide resulted in a change of the dominating organism in the extraction. The Gram positive coccus *Saccharococcus thermophilus* was replaced by a *Lactobacillus* sp. The temperature was found to be important in combination with hydrogen peroxide. Thus, it was necessary to keep the temperature at 70–72°C or above, to be able to suppress the activity of the microflora.

C. Ethane peroxoic acid

Ethane peroxoic acid is an effective disinfectant (48, 76). It is mainly used as a surface disinfectant. In the sugar industry Hercik and Dachovsky (26) have tested it during the extraction and in the beet washing step.

Because ethane peroxoic acid is a dangerous and unstable chemical it was used in low concentrations and in combination with hydrogen peroxide (IV).

D. Mixtures of hydrogen peroxide and ethane peroxoic acid

In this investigation ethane peroxoic acid was not used alone. It was mixed with hydrogen peroxide. Three different mixtures were included in the study: Ekarox B 1,

Ekarox B 5 and Ekarox B 10 (IV). Ekarox B 10 was the most effective of these based on laboratory results (IV) and was therefore tested in full scale experiments. It was used as a shock chemical. The dominating species in the extraction was the same as when hydrogen peroxide was used alone, i.e. a *Lactobacillus* sp. The dominating species was selected by the hydrogen peroxide in the Ekarox B 10. The coccus, *Saccharococcus thermophilus* was sensitive to the Ekarox B 10 and was suppressed. It was replaced by the *Lactobacillus* sp within 48 hours.

Ekarox B 10 showed the same susceptibility to temperature as hydrogen peroxide. In the range of 65–68°C it was hard to suppress the microbial activity but at 70–72°C or above the effect was good.

E. Dithiocarbamates

The dithiocarbamates represent a group of very potent disinfectants. Some of these have been tested in the sugar industry (33, 55, 58). In this study three different dithiocarbamates were tested: Busan 881, Nalco 247 and Antiformin DMT. In the laboratory screening test they were found to be effective disinfectants (IV). However, due to the chemistry of the dithiocarbamates, they are not allowed to be added to the sugar process above a certain concentration. No residuals must be found in the final product. The dominating species of the microflora, both *Saccharococcus thermophilus* and the *Lactobacillus* sp, were not changed to other species by the addition of dithiocarbamates, Busan 881 and Antiformin DMT alone. This was due to the low concentrations, 7–25 ppm, used. Both continuous and periodical addition gave the same result. When the dithiocarbamates were used in combination with shock chemicals, the dominating species changed depending upon the shock chemical used.

The effect on the microflora of the dithiocarbamates was limited and they could not give an acceptable disinfecting result by themselves. Hollaus et al (33) got the same result for Busan 881, but Antiformin DMT showed a better result.

In this study the needed amounts of shock chemicals could be lowered considerably when they were used in combination with the dithiocarbamates. The amounts of formaldehyde and Ekarox B 10 could be reduced by 50 % (IV).

The temperature influenced the effect of the dithiocarbamates both with and without a supporting shock chemical. The effect increased with increasing temperature. However, the dithiocarbamates alone could not even at high temperatures, 70–72°C, suppress the microbial activity.

Thus, the conclusion in this study is that the dithiocarbamates, due to the low concentrations recommended, can serve as supporting disinfectants to a shock chemical but are not suitable as sole disinfectants.

F. Quaternary ammonium compounds

Many different quaternary ammonium compounds have been tested in the sugar industry, mainly at laboratory scale (21, 54, 55, 62, 82). In these studies some compounds have been found to be very effective e.g. Deosteril (55) and Kebosan NL (62), but full scale tests have not been reported.

The anti-microbial effect of the quaternary ammonium compounds has been found to be variable and many parameters influence the effectivity, especially temperature and pH (54). Mixtures of quaternary ammonium compounds and formaldehyde, Anios B×5 and Anios DIF, have been tested (16, 50), but they were not found to be more effective than quaternary ammonium compounds themselves (54).

Due to the reported variation in effectivity and the earlier results from full scale tests in a sugar factory in Sweden (17) quaternary ammonium compounds were not included in this study.

G. Iodine compounds

Iodoacetone has been tested by several authors (24, 53, 54, 84). It is a very effective disinfectant. Guérin et al (25) reported that Septosol I 31 was %000 times more effective than formaldehyde at comparable concentration. Iodoacetone strongly irritates the eyes (86) and is therefore not suitable for use in factories as a disinfectant. For this reason iodoacetone was not included in this study.

Kammerer (36) used an iodophore for beet disinfection during beet washing and noted a considerable reduction of the microflora. In this iodophore the iodine was included in a polyglycoether-iodine complex. Nickisch and Mauch (62) tested an iodophore called Kebozan Z 1. The effect was comparable to formaldehyde. These products were not commercially available.

H. Other disinfectants

Many different substances have been tested in order to determine their disinfecting capacity and suitability for use in the sugar industry. Oldfield et al (63) have used sulphur dioxide. Badgley (2) proposed the use of esters of p-hydroxy benzoic acid. Matteuzzi et al (55) tested a substance called RH-886 (5-chloro-2-methyl-4-isothiazolin-3-one calcium chloride (75 %) and 2-methyl-4-isothiazolin-3-one calcium chloride (25 %). The effect of this complicated disinfectant was about the same as for formaldehyde. Nickisch and Mauch tested 15 different disinfectants in a laboratory study (62). They used there disinfectants with a cationic substance as the active substance (Struktol DMM, Struktol DMK, Kebosan 2771), a cresol (Antiseptol) and an amphotenside (Kebosan 2568). All of these, except Antiseptol, were found to be more effective than formaldehyde.

These products were not commercially available.

I. Temperature

The temperature is a very important parameter in disinfection. Most disinfectants show an increased effectivity with increasing temperature (80, 81). The temperature alone influence composition of the microflora and its activity. There is a marked decrease in the number of living micro-organisms at 74°C compared to 65–68°C during the extraction. The results (I, IV) indicate that few micro-organisms are able to grow in the extraction system at high temperatures. The dominating species found (III, IV) have an optimum temperature of 68–70°C and show a decreased growth

and activity at higher temperatures. For these reasons a temperature of 74°C or above is to be recommended from a microbiological viewpoint. However, for other reasons, such as the quality of the beet, the size of the cossettes and other technical reasons this is not always possible. Moreover, at too high extraction temperature pectine is extracted, which later in the process causes clogging of the filters.

In some sugar factories temperature is used to pasteurize the press water before it is returned to the extraction. The press water is treated in heat exchangers at 95°C which is sufficient to damage most vegetative bacterial cells.

In conclusion, the temperature used in the extraction must be a compromise between microbiological and technical factors. If the temperature must be lower than 74°C, this has to be compensated for by an increased use of chemical disinfectants to suppress microbial activity.

J. Alternating use of two disinfectants

The alternating use of two disinfectants was found to be advantageous (IV). This has also recently been proposed but not tested by Matteuzzi et al (55) and Nickisch and Mauch (62).

The growth and activity of the dominating species will be disturbed when the environment change. In this study formaldehyde and hydrogen peroxide were used alternately. The disinfectant was changed after about 24–48 hours when the respective dominating organism developed. As mentioned above *Saccharococcus thermophilus* and the *Lactobacillus* sp replaced each other when the disinfectant was changed. Also in this system the temperature was important. The growth and activity of the micro-organisms decreased when the temperature was high 72–74°C. This resulted in longer intervals between the changes of disinfectant.

A disinfection system using more than one disinfectant is not limited to the use of two, in fact three or even more may be used. Further on, different combinations or sequences of addition can be used.

Generally, a system with more than one disinfectant, where the action of the disinfectants is different is beneficial. Formaldehyde and hydrogen peroxide act in different ways: formaldehyde forms methylene bridges in proteins and hydrogen peroxide oxidizes various chemical groups. Ethane peroxic acid also oxidizes.

It will be more difficult for a micro-organism to grow and eventually dominate in the extraction vessel since the environment changes with the disinfectant used.

K. Alternative disinfectant to formaldehyde

The aim of this investigation of different disinfectants and combinations of disinfectants (IV) was to find an alternative to formaldehyde.

The result was, that Ekarox B 10 as a shock chemical could be an alternative to formaldehyde, providing that the temperatures was kept at 70–72°C or above. Ekarox B 10 in combination with Busan 881 may also be used with the same temperature criteria as for Ekarox B 10 alone (IV).

Again the temperature must be emphasized as a very important parameter concerning disinfection and it is important to know the actual temperature in different parts of the extraction vessel. If the temperature is too low, the disinfection can be insufficient and the amounts of disinfectant used will be unnecessarily large.

7. Taxonomy and characteristics of thermophilic bacteria in the extraction

The beet sugar extraction is a thermophilic system which operates continuously and has a temperature of 70°C. In this system only thermophilic bacteria will grow. Further on, only those organisms which have a generation time shorter than the dilution rate can become dominant in the system.

Psychrophilic and mesophilic bacteria may survive the transport through the extraction vessel. They have either very low or no metabolic activity and will therefore not cause losses in the extraction, although they can be present in large numbers.

The dominant organisms in the extraction are most interesting since they cause most damage and sucrose losses.

Important bacteria are reported to be species of the genus *Bacillus*, especially the *B. stearothermophilus* group. This organism is, by many authors, considered to be the only important organism in the extraction (1, 20, 27). In tower extractions species of the genus *Clostridium* are reported e.g. *Cl. thermohydrosulphuricum* and *Cl. thermosaccharolyticum* (31, 45). Other species of the genus *Bacillus* which have been isolated from the extraction and can grow in the thermophilic temperature range are *B. sphaericus*, *B. coagulans*, *B. subtilis* and *B. licheniformis* (19, 27, 43). None of these *Bacillus* spp have been reported to dominate in the extraction. No other types of bacteria have been reported to dominate in the extraction vessel.

All the organisms described in the literature as dominant organisms are spore formers. In spite of the fact that about 35 % of the organisms isolated were spore formers (II), no spore former was recorded as being dominant.

In this investigation the normally dominating organism found was *Saccharococcus thermophilus* (III). When hydrogen peroxide was used as a disinfectant, a thermophilic *Lactobacillus* sp dominated. (IV). Thus, both the dominating organisms were non-spore formers and Gram positive. They have not been reported earlier. The organisms could not be isolated from frozen samples of extraction juice.

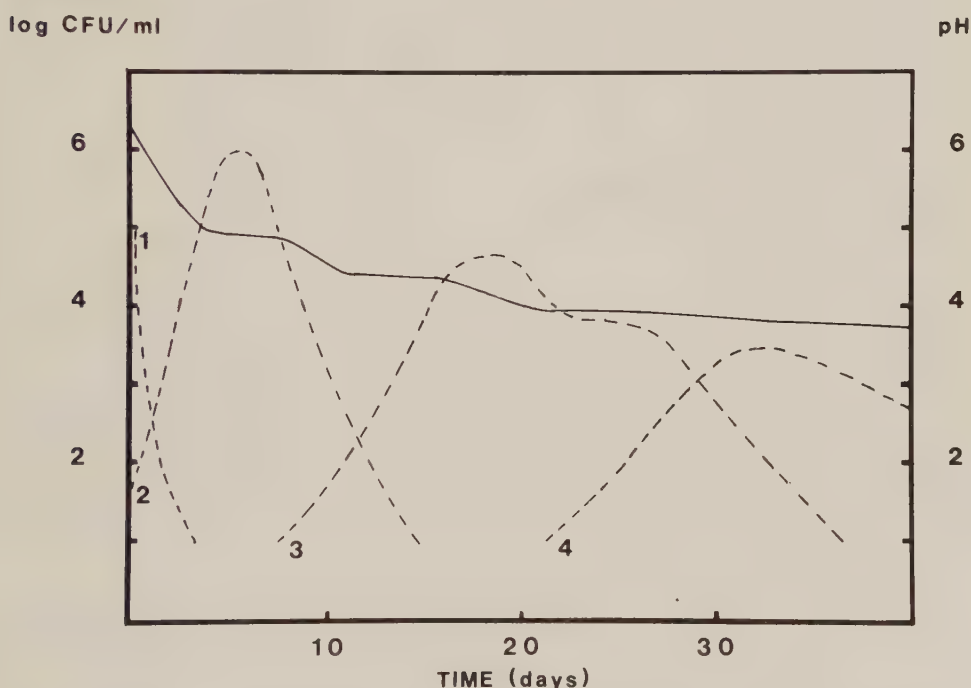
It was interesting to note that *Saccharococcus thermophilus*, which produced extracellular catalase, was very susceptible to hydrogen peroxide disinfectants while the *Lactobacillus* sp, which did not produce extracellular catalase, was tolerant to rather high concentrations of hydrogen peroxide. One explanation may be that the *Lactobacillus* sp had an intracellular catalase.

Gram positive organisms constituted 80 % of the isolates in this investigation (II) as well as all dominating species. Other investigators have also found more Gram positive organisms than Gram negative ones (79). It seems that the conditions in the extraction are more favorable to Gram positive organisms compared to Gram negative i.e. temperature, sucrose concentration and disinfectant used. Thermophilic bacteria are taxonomically not concentrated to certain genera, but they occur in almost all groups of bacteria (7, 14, 77). It is possible to isolate thermophilic organisms from all environments and not only from hot springs and other wellknown thermophilic environments. The thermophilic organisms detected in this study (II) originate from the soil adhered to the cosettes. The genus *Bacillus* constituted 35 %, Gram positive non spore forming thermophilic rods, including the dominating

species of the genus *Lactobacillus* and the coryneform group of bacteria, constituted 40 % and Gram negative rods 18 % of the isolates. Among the Gram negative organisms the genus *Thermus*, *Pseudomonas*-like organisms and *Flavobacterium*-like organisms were found. The group of Gram positive cocci was 5 % and was made up mainly of the new genus *Saccharococcus*. The actinomycetes constituted 2 % and were characterized as *Streptomyces* sp. The organisms isolated under anaerobic conditions were all characterized as *Clostridium* spp.

In different process steps e.g. extraction, press water system, raw juice cistern and preliming. Gram positive non-spore forming rods were isolated and recorded as dominating species. All of them were facultatively anaerobic and produced L-lactic acid as the main metabolite of sucrose degradation. The organisms were all different and most of them were characterized as thermophilic *Lactobacillus* sp. The total environment in a certain process step is different from the environments in all other process steps. Each process step has its own physical and chemical conditions and consequently also its own microflora and dominating species.

FIGURE 8.



Succession of different species in a batch culture of extraction juice. Temperature: 70°C. Time: 40 days.

A laboratory experiment. Cultivation method: See (I).

pH:

Saccharococcus thermophilus:

Gram positive non-spore forming rod:

Bacillus sp:

Bacillus sp:

—————
1 -----
2 -----
3 -----
4 -----

The reason that many of the dominating organisms were taxonomically found to be in the same group of bacteria is probably that the environments in these process steps have some physical and chemical characteristics in common, e.g. oxygen content, pH and sucrose concentration. However, the conditions are not identical and therefore it is not likely that the same organism will become the dominant species in different process steps.

A laboratory experiment was performed in order to study the succession of different organisms in a batch culture of extraction juice. A batch of 10 litres of extraction was used. The temperature of the juice was kept at 70°C during the experiment. The batch was run for 40 days. The results are shown in Figure 8. At the time of sampling *Saccharococcus thermophilus* dominated but it rapidly disappeared in the batch culture and a Gram positive non-spore forming rod was the successor. The two following organisms were species of the genus *Bacillus*, but they did not belong to the *B. stearothermophilus* group. When the *Bacillus* sp began to dominate, the pH was 4.5 and below. It was interesting to note that the growth of *Bacillus* sp started late, after about one week. At that time were the conditions in the batch culture were quite different from those in the extraction.

Analysis of the similarity between the isolated thermophilic bacteria (II) showed that the most homogenous group was the 70°C Gram positive cocci. In all other groups the organisms were less closely related to each other.

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I

SIZE AND ACTIVITY OF THE MICROFLORA IN BEET SUGAR EXTRACTION

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Summary

The distribution, size and composition of the microflora in Swedish beet sugar extraction are described. Three methods, acridine orange staining, fluorescein diacetate staining and determination of colony forming units, were used to measure the size of the microflora. A comparison of different agar media for aerobic and anaerobic microorganisms was carried out in order to find the most suitable media.

Two different systems of extraction, the DDS extraction trough and the Buckau-Wolf extraction tower, have been compared in respect to the microflora and their activity. The number of bacteria in trough extraction and in tower extraction was found to be quite similar if the extraction temperature exceeded 71°C. But if the temperature was below 69° the number of bacteria in the DDS trough increased considerably due to the growth of a Grampositive thermophilic coccus. To our knowledge this coccus has not been found in either beet extraction or elsewhere.

Commonly the *Bacillus* strains have been considered the most troublesome microorganisms in beet sugar extractions but we have never observed any spore forming bacteria being dominant. Instead we have found the thermophilic coccus in all Swedish sugar factories during all the four years of this study.

Introduction

The sugar from sugar beets is normally extracted by a countercurrent extracting system in hot water. Although the temperature in the process is about 70°C it is possible to isolate large numbers of bacteria from the extraction system.

Most of the earlier investigations of the microbial flora in the beet sugar extraction have been performed with bacteria isolated from samples stored for shorter or longer times. Sometimes the samples have not been analysed until after the campaign.

According to many authors, *Bacillus stearothermophilus* is the most important bacterium in beet sugar extraction^{1, 5, 6, 12, 15, 17, 19}. DEMETER et al⁶ have found more than 10⁹ *B. stearothermophilus* cells/ml in an extraction tower without the addition of formaldehyde during 27 hours. However, KLAUSHOFER and PARKKINEN¹⁸ have shown that under anaerobic conditions, the number of *Clostridium thermohydrosulfuricum* cells was about 2·10⁷/ml in Austrian extraction

towers, while the amount of *B. stearothermophilus* was only about $4 \cdot 10^4$ /ml. The clostridia grew mainly in the upper part of the tower where the amount of oxygen was very low. In raw juice as well as in the press water the number was small.

Some authors have measured the total numbers of bacteria in the extraction. WEIDENHAGEN²⁷ has found about $3 \cdot 10^8$ cells/ml in a tower by Congo red staining. However, the maximum numbers obtained by CORNET³ and by GUERIN et al¹¹ were about $5 \cdot 10^6$ cells/ml.

DZIENGEL and MAUCH have in three articles^{8, 9, 10} described experiments on microbial growth and sugar degradation. However, most of their work was performed with pure strains of *Bacillus* (probably *B. stearothermophilus*) in laboratory fermentors. Some of the bacteria were isolated in sugar factories and some were obtained from culture collections. Under extreme conditions, they found microbial generation times as low as 15 minutes⁸.

During the extraction the microbial activity cause losses of sucrose, and it is held in sugar industry that the losses are between 0.05 and 0.5 % of the weight of the beet^{4, 7, 14, 24}. The main degradation product is L-lactic acid which has been shown in several investigations.

VERHAART and de VISSER²⁶ reported that during periods with "strong infections" the concentration of L-lactic acid in raw juice increased to about 650 mg/l. van der POEL²⁵ also found that in a strongly infected extraction the concentration was about 600 mg/l but in a moderately infected extraction the concentration of L-lactic acid was 100–200 mg/l. SCHIWECK and BÜSCHING²² analysed the raw juice enzymatically once a week. They found that the mean values for the 1969/70 year campaign were 35.9 mg L- and 10.7 mg D-lactic acid per 100 g sugar. This corresponds to about 54 and 16 mg/l respectively.

OLDFIELD et al²¹ have semiquantitatively assayed the concentrations of lactic acid with thin layer chromatography for many years. During the three campaigns 1976–1978 they used gas liquid chromatography for a more accurate determination of the lactic acid concentrations. They analysed samples taken every second week. The concentrations in 17 factories during the studied period ranged from 21 to 245 mg/l with a mean of 109 mg/l.

In all the investigations concerning the microbial activity in sugar extraction it is supposed that the bacteria directly or indirectly convert the sucrose to L-lactic acid. The only exception to this is the investigations by WINSTRÖM-OLSEN et al^{28, 29} who found no detectable loss of sucrose. However, the concentration of glucose decreased and the concentration of fructose was almost constant. They assumed that the decrease in concentration of glucose depended upon the fact that the microorganisms consumed the glucose only. But as they did not determine the number or activity of the microorganisms in the extraction we do not think it is possible to make such a conclusion.

The present investigation was carried out in order to study the number, composition and activity of the microflora in the beet sugar extraction. Five different sugar factories were studied during four campaigns 1977–1980. Two of the factories, one with DDS extraction and one with Buckau-Wolf extraction tower, were studied more in detail. In this paper the size of the microflora and its activity in the sugar extraction are described. In a forthcoming paper the composition of the microflora and its characterization are dealt with.

Material and methods

Substrates

The following nine substrates were examined in respect to growth of bacteria under aerobic conditions:

Tryptone Glucose Extract Agar (Oxoid) with the addition of sucrose 5 g/l (designated TS)

Tryptone Glucose Extract Agar (Oxoid) (designated T)

Sucrose Peptone Agar² (designated SPA)

Dextrose Tryptone Agar (Oxoid) (designated DTA)

Nutrient Agar (Oxoid) (designated NA)

Nutrient Agar (Oxoid) with the addition of sucrose 5 g/l (designated NAS)

Tryptone Soya Agar (Oxoid) with the addition of sucrose 5 g/l (designated TSAS)

Plate Count Agar (Oxoid) with addition of sucrose 5 g/l (designated PCAS)

Blood Agar Base (Oxoid) with addition of sucrose 5 g/l (designated BABS)

The pH of all the substrates was 6.8 ± 0.2 . Sterilization was carried out by autoclaving 20 min at 121°C.

The following four substrates were examined with respect to the growth of bacteria under anaerobic conditions:

Trypticase Sucrose Iron Agar (designated TSE)¹⁸

Reinforced Clostridial Agar (Oxoid) with the addition of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0,05 g/l (designated RCA-Fe)

Iron Sulphite Agar (Oxoid) (designated ISA)

Tryptone Glucose Extract Agar (Oxoid) with the addition of sucrose 5 g/l and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05 g/l (designated TS-Fe)

The substrates were melted and distributed in test tubes 16×160 mm, 10 ml in each and autoclaved 20 min at 121°C. Before inoculation the tubes with the agar were melted, then boiled for 5 min and cooled to 60–65°C. Immediately after inoculation the tubes were placed in cold water to solidify.

The examination of both aerobic and anaerobic growth was carried out on samples taken at five different factories. The samples did not contain any dominating bacteria. The agar plates were incubated at 35°, 55° and 70°C.

For the determination of the number of fungi the Sabouraud-dextrose agar (Oxoid) was used. The petri dishes were incubated at 25°C for 72 h.

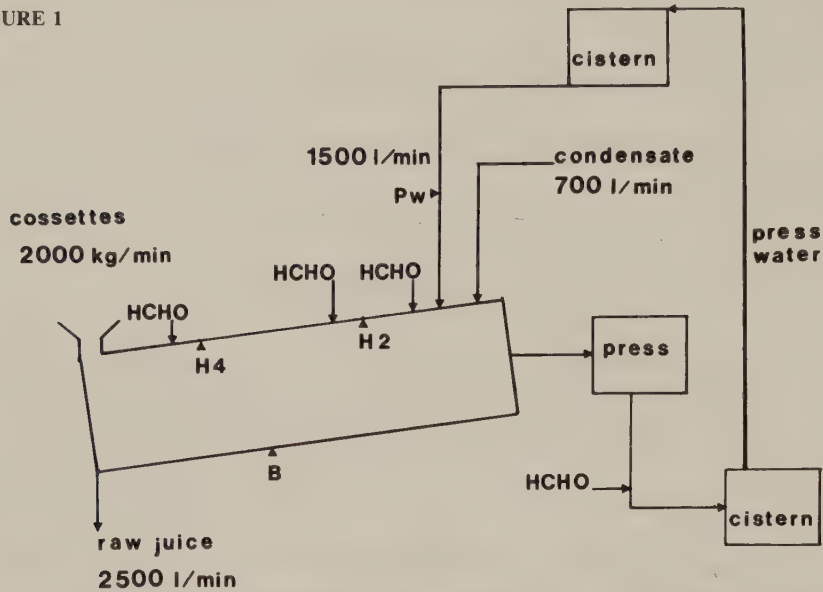
Sampling and incubation

In Figures 1 and 2 schematic plans of a DDS, extraction trough system and a Buckau extraction tower system are given. The points of sampling are indicated in the figures as well as the size of the material flow through the systems. The effective volumes of the different parts of the systems can also be seen in the figures.

During the campaigns samples were taken almost once a day from the two DDS-troughs at the Örtofta sugar factory. At the Jordberga sugar factory samples were taken from the tower at least once a week. During periods of special interest, e.g. when the number or activity of the bacteria were extremely high, the samples

were taken much more often in both factories. To avoid varying results depending of different concentrations of HCHO, all samples were taken three hours after the

FIGURE 1

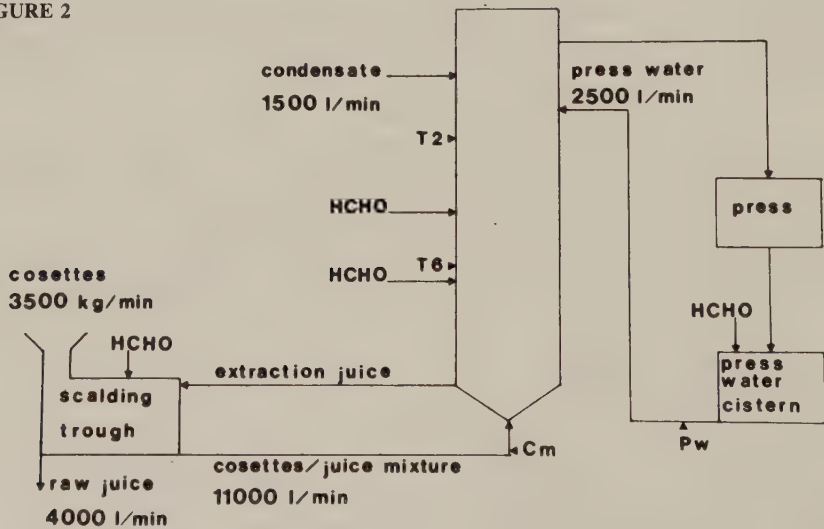


The beet extraction plant in the sugar factory in Örtofta consists of two DDS troughs, each with an effective volume of 280 m³. The press system, which contains two vessels with a total volume of 20 m³ is common for both the troughs.

The size of the material flow is given. The places of sampling are indicated as follows: H2=hatch 2, H4=hatch 4, B=bottom tap, Pw=press water.

The addition points of formaldehyde (HCHO) are indicated by arrows.

FIGURE 2



The Buckau-Wolf extraction tower at the sugar factory in Jordberga. The effective volume of the tower is 430 m³, of the scalding through 60 m³ and of the press water system 25 m³.

The size of the material flow is given. The places of sampling are indicated as follows: T2=tap 2, T6=tap 6, Cm=cossettes/juice mixture, Pw=press water.

The normal addition points of formaldehyde (HCHO) are indicated by arrows.

addition of HCHO except in investigations where the importance of the HCHO-concentration was studied (Figure 5, 6 and 7).

The samples were diluted immediately after sampling and spread for the determination of the CFU number. The plates and the tubes were incubated at 35, 55 and 70°C during 48 hours.

Fluorescein diacetate staining

The method described by SÖDERSTRÖM ²³, modified by LUNDGREN ²⁰ was used. Immediately after sampling the extraction juice was stained, filtered through a dyed 0.22 μm Millipore filter ¹³ and then examined under a Reichert Zetopan microscope with incident UV-light (Binolux HBO 200 Hg lamp).

Acridine orange staining

Twentyfive ml of the extraction juice were mixed with acridine orange (BDH Chemicals) at a final concentration of 67 $\mu\text{g/ml}$. After five min the sample was filtered through a dyed 0.22 μm Millipore filter ¹³ and examined in the same way as the fluorescein diacetate preparation.

Gas chromatography

For the detection of metabolic products in the extraction, gas chromatography was used. A sample of four ml was acidified with 0.5 ml 25 % (w/v) metaphosphoric acid. The resulting pH was about 2. To remove particles, the sample was centrifuged 20 min at 1800 $\times$ g and filtered through a Millipore 0.22 μm filter. The samples were stored in glass ampoules until being analysed. The analysis was carried out on a Pye Unicam 104 gas chromatograph with a glass column with an inner diameter of 4 mm and a length of 160 cm. The column packing material used was Chromosorb 101 80/100 mesh (Johns Manville). The gas chromatograph was run with a temperature program of 130°–230°C with an increase of 10°C/min and finally isothermally at 230°C for 5 min. For the detection, a flame ionization detector (FID) was used.

With this method of analysis lower fatty acids (C_2 – C_6), alcohols (C_1 – C_5), aldehydes (C_2 – C_5), ketones (C_3 – C_6) and glycols (C_2 – C_4) were detected.

Enzymatic analysis of lactic acid, glucose and fructose

L-lactic acid, D-lactic acid, fructose and glucose were determined by enzymatic analysis. The test kits used were manufactured by Boehringer Mannheim GmbH Diagnostica, Western Germany, catalogue number 256773 and 139106.

Redox potential measurements

The redox potential in the sugar extraction was measured with a Philip M-13 platinum electrode and an Ingold 360-T silver/silver-chloride reference electrode. The platinum electrode was placed directly into the bottom of the extraction trough B, near the bottom valve (Figure 1) whereas the reference electrode was placed in an electrode vessel and connected to the extraction with a salt bridge of 3 M KCl.

Results

Test of substrates

The results of the examination of aerobic growth on the nine agar substrates are shown in Table 1. The substrate TS gave the highest number of CFU/ml for all of the three different temperatures investigated. This substrate also gave the greatest variety of different colony types for the three temperatures. Attempts were also made to use the extraction juice as a substrate, but this failed, probably due to the great changes (precipitation and oxidation) when the juice was taken out from the extraction and sterilized. Also the formaldehyde present can perhaps prevent growth.

TABLE 1 *Test of nine different substrates for aerobes at 35, 55 and 70°C*

	SAMPLE	TS	T	SPA	DTA	NA	NAS	TSAS	PCAS	BABS	CFU/ml
		% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	% CFU	100 %
35°C	A	100	85	15	5	40	53	10	12	12	9.5×10^3
	B	95	100	8	1	28	40	16	5	20	7.0×10^3
	C	100	92	29	9	52	62	23	10	10	2.6×10^3
	D	100	80	5	2	29	31	8	6	13	8.5×10^3
	E	100	93	2	1	23	39	20	17	17	10.0×10^3
55°C	A	95	100	5	11	10	15	20	10	1	9.5×10^2
	B	98	100	16	28	6	8	8	14	1	7.0×10^2
	C	100	72	7	34	2	2	3	15	5	25.0×10^2
	D	100	90	5	5	2	5	11	12	2	30.0×10^2
	E	100	73	5	22	7	5	16	20	3	20.0×10^2
70°C	A	100	90	3	35	6	8	5	14	5	1.1×10^2
	B	96	100	5	41	8	12	0	19	3	1.4×10^2
	C	100	87	10	29	3	10	7	23	2	2.5×10^2
	D	90	100	12	25	3	19	9	7	1	1.5×10^2
	E	100	95	7	17	5	14	5	10	1	2.0×10^2

The results of the examination of anaerobic growth on the four substrates are shown in Table 2. The ISA substrate gave the best result in having the highest number of CFU/ml at the three temperatures investigated. The TS-Fe substrate (which was almost the same as that normally used for aerobes) gave a low number. In

the case of the anaerobes, attempts were also made to get a substrate of extraction juice, but these failed for the same reasons as for the aerobes.

The CFU-number obtained on the TS and ISA substrates is not the total number of aerobic and anaerobic bacteria. However, our opinion is that we have found all the bacteria which dominate over any other. This conclusion is drawn from the following fact. Every time large amounts of bacteria could be detected either directly in the microscope after staining (Cf Figure 6) or indirectly by analysis of the production of metabolites it was also possible to grow these dominating bacteria on the substrates chosen.

TABLE 2 *Test of four different substrates for anaerobes at 35, 55 and 70°C*

	SAMPLE	ISA	RCA-Fe	TSE	TS-Fe	CFU/ml
		% CFU	% CFU	% CFU	% CFU	100 %
35°C	A	100	10	73	42	7.0×10^2
	B	100	16	82	37	5.3×10^2
	C	100	21	67	32	4.1×10^2
	D	95	8	100	51	10.0×10^2
	E	100	12	85	29	5.0×10^2
55°C	A	100	7	80	50	2.0×10^2
	B	100	15	90	60	9.5×10^2
	C	100	5	75	36	5.2×10^2
	D	100	10	68	13	8.5×10^2
	E	100	5	65	28	4.3×10^2
70°C	A	95	0	100	16	0.5×10^2
	B	100	5	59	10	0.7×10^2
	C	100	10	77	5	1.1×10^2
	D	100	12	52	5	1.0×10^2
	E	100	18	84	15	0.8×10^2

Choice of incubation temperature

We have performed experiments to show if bacteria isolated after growth on agar plates at one of the temperatures 35, 55 and 70°C would also grow at any of the other temperatures. The results showed that bacteria isolated at 35° could not grow at 55° and vice versa. A very low number of the "55°-bacteria" could grow at 70°, but the "70°-bacteria" grew so slowly at 55°C that they could not generate any colonies during the incubation time used.

Microbial flora and its activity in DDS troughs

The extraction in the sugar factory in Örtöfta is made in two DDS troughs. Under the studied period one of these had a normal extraction temperature of about 71°C. For

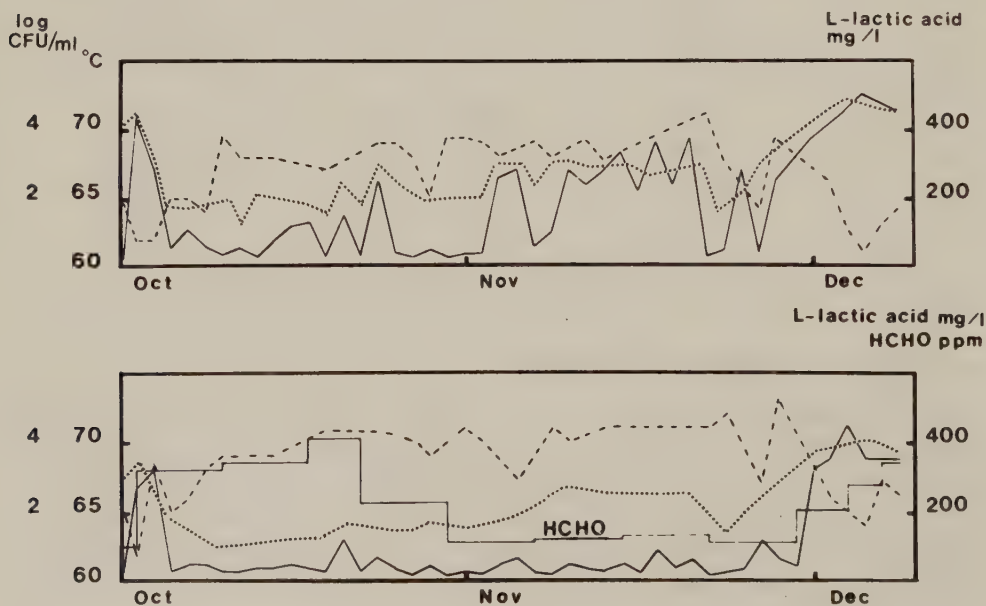
technical reasons the temperature in the other was a few degrees lower during the campaigns 1977–1979. This difference made Örtöfta very useful for the investigation of the influence of the temperature on the microbial flora and its activity.

In Figure 3 the results in Örtöfta from the campaign of 1978 are shown. During the first week a great number of bacteria and a high activity was found. When the formaldehyde concentration and the temperature were increased both size and activity of the microbial flora decreased. After about four weeks the HCHO concentration was stepwise decreased from more than 300 ppm to about 100 ppm. This caused an increase in the number of CFU in both troughs, but the concentration of L-lactic acid increased only in trough A.

During the period of high HCHO concentration, the microflora consisted of at least 50 different bacteria according to their colony morphology on agar substrates. None of these bacteria dominated over any other. However, when the HCHO concentration was decreased, one organism – a Gram positive thermophilic coccus – became dominating in trough A. The CFU number of these bacteria was nearly 10000 per ml. but as these bacteria are growing in aggregates of 50–100 cells the real number was 10^5 – 10^6 cells per ml. This bacterium was also found in trough B during this period, but normally not in CFU numbers exceeding 50/ml.

At the end of the campaign the extraction temperature decreased for technical reasons. Then the size of the microflora as well as the concentration of L-lactic acid increased considerably. The previously mentioned thermophilic coccus became dominating in both troughs.

FIGURE 3



The size and activity of the microflora in the two DDS troughs at the sugar factory in Örtöfta. All samples were taken from the bottom taps three hours after the addition of HCHO.

The concentration of HCHO was the same in both troughs but is indicated only in trough B.

Other legends:

--- temperature, number of CFU/ml, _____ concentration of L-lactic acid.

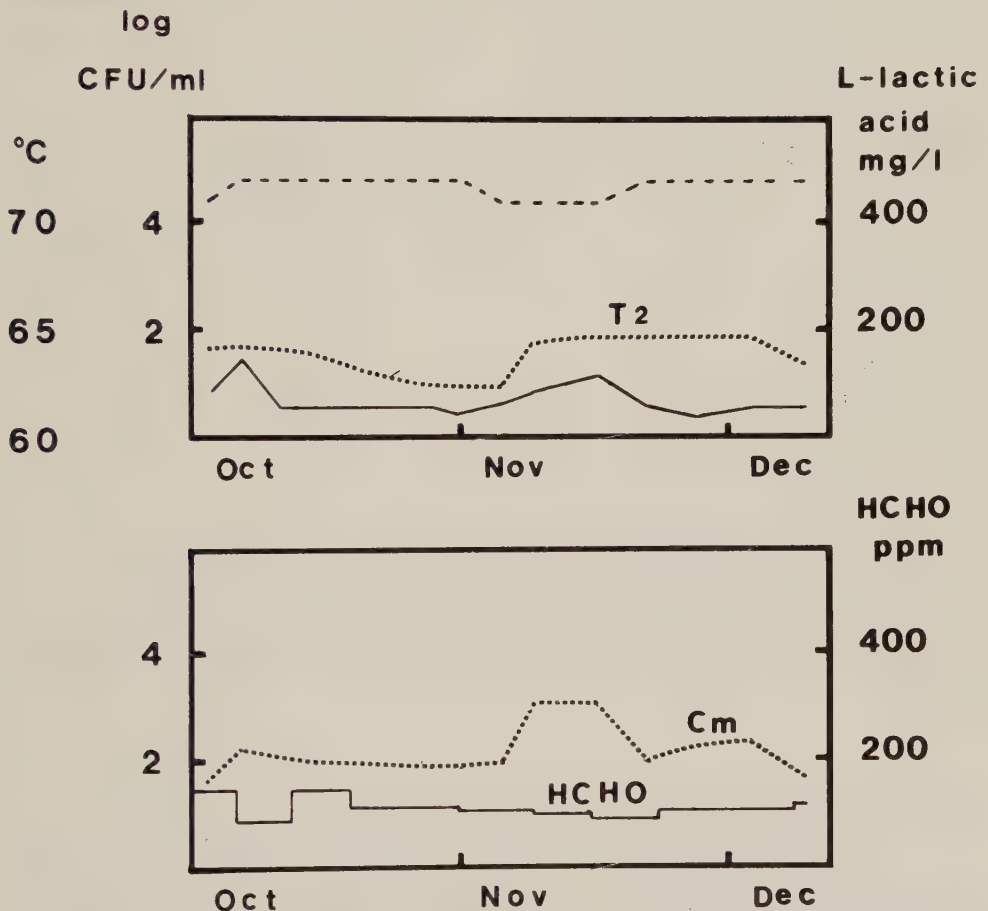
During the whole campaign the number of organisms and the concentration of L-lactic acid was considerably higher in trough A than in B. This was due to the temperature difference between the two vessels.

Similar results as in 1978 were obtained in 1977 and 1979. However, in 1980 the heating system was changed in such a way that the temperature was equal in the troughs. This change made the number and activity of the bacteria the same in both troughs during this campaign.

Microbial flora and its activity in a Buckau-Wolf tower

In the extraction tower at Jordberga (Figure 4) there was without exception a smaller number of bacteria and a lower concentration of L-lactic acid than those found in Örtofta. This can be explained by the high temperature (71–72°) and the even running during the whole campaign.

FIGURE 4



The size and activity of the microflora in the Buckau-Wolf extraction tower at the Jordberga factory. The samples were taken from tap 2 three hours after the addition of HCHO.

Legends: --- temperature, number of CFU/ml in the tower (T2) and in the cosette/juice mixture (Cm), ——— Concentration of L-lactic acid and of HCHO respectively.

During the second and third weeks of November the temperature in the tower decreased one to two degrees. This caused an increase in the number of CFU, as well as an increase in the concentration of L-lactic acid. At the same time the thermophilic cocci became dominant in the scalding trough.

In the scalding trough the number of CFU was higher than in the tower because of the temperature gradient of 50 to 74°C. This gradient allowed the thermophilic coccus to grow and to dominate during some periods. However, we could never isolate this bacterium in the tower not even from the tap nearest the cossette inlet.

The number of bacteria during "normal" conditions

As seen in Figures 3 and 4 the number of bacteria in trough extractions and in tower extraction are quite similar, if there is no dominating bacteria present. In fact this similarity was valid for all factories during all four years of this study. Neither differences in weather, soil, amount of clay on the beets, nor extraction system caused any divergence in the numbers of bacteria in extraction juice (Table 3). If, however, the extraction temperature went down under 68°C the thermophilic coccus began to dominate in the DDS trough.

TABLE 3 *The size of the microflora*

		RAW JUICE		EXTRACTION JUICE	
		No dominating bacteria	Dominating bacteria	No dominating bacteria	Dominating bacteria
35°C	aerobes	10^5-10^6	10^5-10^6	10^3-10^4	10^3-10^4
	anaerobes	10^4-10^5	10^4-10^5	10^2-10^3	10^2-10^3
55°C	aerobes	10^2-10^3	10^3-10^4	10^2-10^3	10^3-10^{4*}
	anaerobes	10^2-10^3	10^2-10^3	10^2-10^3	10^2-10^3
70°C	aerobes	10^1-10^2	10^5-10^6	10^1-10^2	10^1-10^{6*}
	anaerobes	10^1-10^2	10^1-10^2	10^1-10^2	10^1-10^2
25°C fungi		$1 \cdot 10^2-5 \cdot 10^2$	$1 \cdot 10^2-5 \cdot 10^2$	0	0

The figures (CFU/ml) are valid both for DDS troughs and for Buckau-Wolf towers during all of the campaigns 1977-1980 in five Swedish sugar factories. The only exceptions are the figures indicated with *. They are valid only for DDS troughs.

The microflora in the raw juice

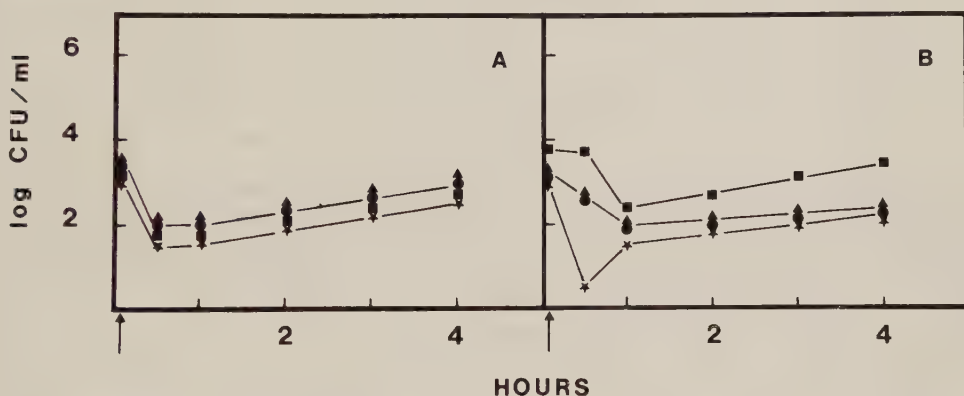
The size and composition of the microflora in the raw juice is not the same as in the main part of the extraction. This depends on the fact that many of the microorganisms are washed off the surface of the beets at the cossette end of the extraction. They follow the raw juice without being heated to more than 35-40°C. We have found about 100 times more mesophilic bacteria in the raw juice than in the extraction juice while the number of thermophiles was the same (Table 3). We have also isolated fungi in the raw juice but never in the extraction troughs or towers.

If there are dominating bacteria growing in the DDS troughs or in the scalding troughs, the number of thermophilic aerobes in the raw juice is the same as in the extraction juice in the DDS troughs. (In the extraction towers we have never found any dominating bacteria growing).

Distribution of microorganisms in the extracting systems

In order to investigate the distribution of microorganisms in a DDS extraction trough (Fig. 5 A) and in a Buckau extracting tower (Fig. 5 B), samples were taken at different places. The results show that the amounts of bacteria in samples taken from the trough at hatch 2 and 4 and at the bottom valve do not differ. The Buckau extraction tower shows the same level of CFU/ml for tap 2 and 6. The press water has a lower content of living bacteria which is due to the fact that the concentration of formaldehyde when just added in the press water system is higher than that in the tower. In the scalding trough the temperature gradient favours a relatively greater microbial growth.

FIGURE 5



The distribution of the microflora in different parts of the extraction systems. The number of the aerobic CFU/ml at 55°C was determined. The addition of 200 ppm HCHO is indicated by the arrows.

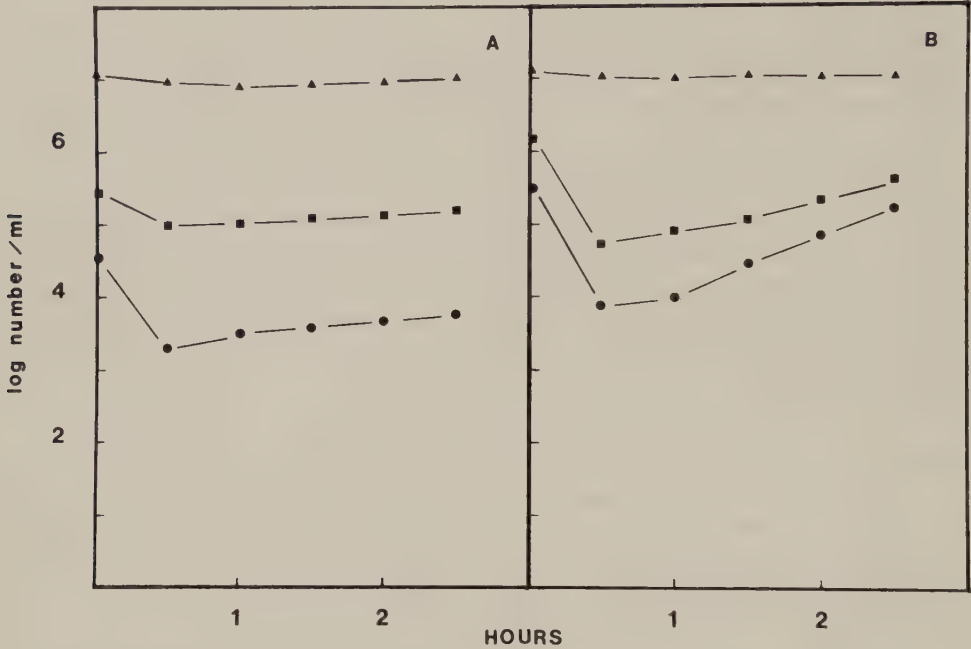
- A. The samples were taken from H2 (●), H4 (▲), B (■) and Pw (★) in trough A at the sugar factory at Örtöfta (cf. Figure 1). The temperature was 70–71°C at the sampling occasion.
- B. The samples were taken from T2 (●), T6 (▲), Cm (■) and Pw (★) in the Buckau-Wolf tower at the sugar factory at Jordberga (cf. Figure 2). The temperature was 71–72°C.

The disinfecting ability of formaldehyde

During our studies we have considered the investigation of the disinfecting ability of HCHO to be of great importance. We have followed the number and activity of the microflora just before the addition of HCHO and during some hours thereafter. In Figure 6 the effect of HCHO is shown on the total number of cells on the FDA active cells and on the CFU. The number of FDA as well as the CFU cells was larger when the dominating cocci were present. After the addition of HCHO the number of living

cells decreased considerably, but after two hours the original level was almost reached.

FIGURE 6



The size of microflora in the sugar extraction.

The samples were taken from the bottom of troughs A and B at the Örtöfta factory. In trough B (Figure B) a thermophilic coccus dominated the microflora but in trough A (Figure A) there was no dominating micro-organism.

At the time=0, HCHO was added to a concentration of 100 ppm.

▲ = the total number of cells (acridine orange staining)

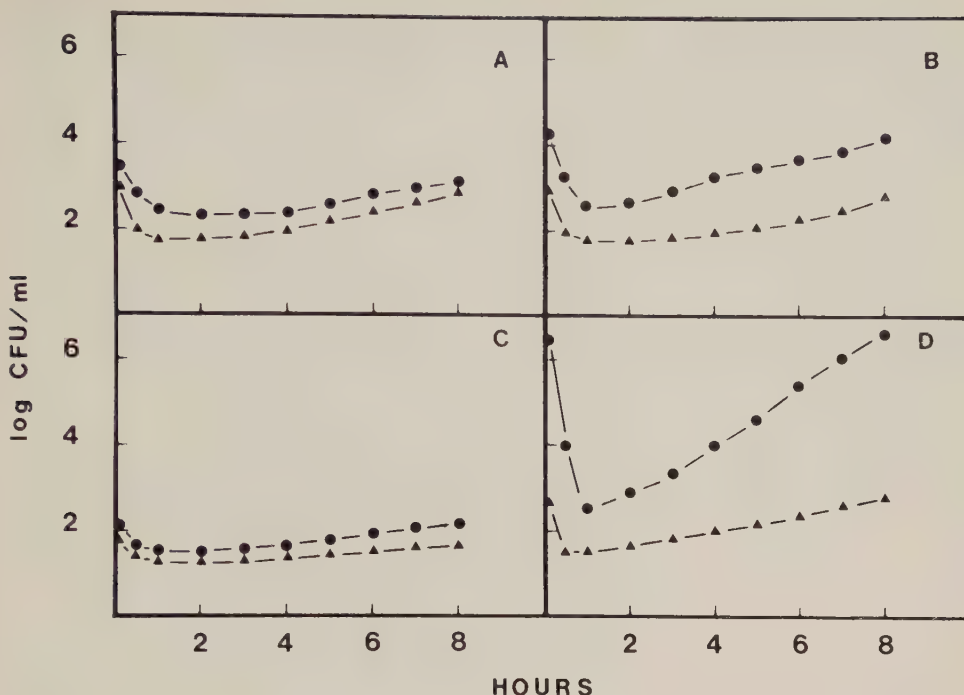
■ = FDA active cells (fluorescein diacetate staining)

● = the total number of colony forming units

(aerobes and anaerobes growing at 35, 55 and 70°C respectively)

In Figure 7 the results are given from an investigation of the change in CFU number of aerobes and anaerobes in the presence of HCHO. The samples in Figures 7 A and C were taken from the bottom valve of the B-trough at Örtöfta during normal conditions without any dominating bacteria. Figures 7 B and D show the results when the thermophilic coccus dominates. These samples were taken in the A-trough at Örtöfta during a period when the temperature was 68°C. During normal operating condition the number of cells growing at 55°C is larger than those growing at 70°C. However, if the coccus dominates, the number of aerobic CFU is larger at 70 than at 55°C. After the addition of HCHO the number of living cocci decrease in one hour from $2 \cdot 10^6$ to $2 \cdot 10^2$. However, the bacteria grow rapidly and after about 7 hours they have reached the original level of $2 \cdot 10^6$ /ml. If the dilution rate of the water phase (1.2 h^{-1}) is taken into consideration, the real growth rate of the bacteria is about 3 h^{-1} which means a generation time of about 20 minutes. A corresponding calculation shows that the generation time for the anaerobes is about 35 minutes at 68°C (Fig. 7 D)

FIGURE 7



The number of micro-organisms growing at 55 and 70°C.

The samples were taken at the Örtöfta factory from the bottom tap of trough B with an extraction temperature of 71–72°C (Figures A and C) and from the bottom tap of trough A, temperature 68°C (Figures B and D). The number of aerobic (●) and anaerobic (▲) colony forming units was assayed at 55°C (Figures A and B) and at 70°C (Figures C and D). At time=0. HCHO was added to a concentration of 200 ppm.

Production of metabolites

During the extraction the bacteria degrade the sugar and produce different metabolites. In Table 4 the amounts of some acids and alcohols are given. Under normal conditions the concentration of L-lactic acid is about 50 mg/l while the other metabolites do not exceed 25 mg/l. This is valid for both trough and tower extractions.

If any bacteria dominate the microflora, and consequently grow in larger numbers, the concentration of sugar degradation products increases considerably. During the period when the number of the thermophilic cocci is at a high level highest (CFU=1,5×10⁶/ml), we have found concentrations of L-lactic acid as high as 1200 mg/l.

TABLE 4 *Amounts of metabolites in DDS-extraction*

Metabolite	mg/l	< 5	5-25	26-50	51-100	101-250	251-500	> 500
L-lactic acid		0	12	15	9	16	10	4
D-lactic acid		30	34	1	1	0	0	0
Acetic acid		44	12	6	3	1	0	0
Propionic acid		44	20	1	1	0	0	0
n-butyric acid		49	16	1	0	0	0	0
Iso-butyric acid		46	14	3	3	0	0	0
n-valeric acid		49	15	1	1	0	0	0
Ethanol		53	9	4	0	0	0	0
Iso-propanol		54	10	2	0	0	0	0
Iso-butanol		54	9	2	1	0	0	0
Acetone		47	18	1	0	0	0	0

The metabolites were analyzed in 66 samples by gas chromatography with the exception of D-lactic acid which was enzymatically analysed. The samples were taken during periods both with and without dominating bacteria. The table shows the number of samples in different concentration intervals.

Discussion

The size and composition of the microflora in the beet sugar extraction could be assumed to depend on the number of organisms which is transported together with the beets into the extraction and/or the physical conditions in the extraction system, e.g. temperature, flow rate, sort and concentration of disinfectant.

During our four years of study we have never been able to find any divergences in the microflora which could be correlated to any variations in wheather and amount of soil in the beets before the extraction. The only exception was found when the extraction temperature for technical reasons had to be decreased to 65–68°C after periods of freezing and thawing. Such a decrease changed both the size and activity of the microflora.

Under ideal conditions the bacteria in the extraction vessels – both the Buckau-Wolf tower and the DDS trough – do not grow and do not have any higher metabolic activity. This is evident from the fact that the size of the microflora is the same in the two extraction systems when there are no dominating bacteria present.

Main factors determining growth of dominant bacteria

We believe that all factors which determine the size and sort of any dominating microflora in the extraction are to be found in the extraction system itself.

Oxygen

One of these factors is the availability of oxygen. The importance of oxygen can be clearest seen in the Buckau tower, where we have never found the thermophilic coccus, though the tower is sometimes fed with these cocci from the scalding trough and the temperature is too low to kill the bacteria. The reason is that the concentration of oxygen is too low for the survival of the bacteria which need a redox potential exceeding -200 mV (in the tower the redox potential is below -300 mV).

In the DDS through, on the other hand, the redox potential exceeds -200 mV which permits growth of the thermophilic coccus.

As the concentration of oxygen is low in the extraction tower, growth of anaerobic bacteria could be expected. That anaerobic sporeforming bacteria actually can grow in towers has been shown by KLAUSHOFER and co-workers^{16, 18}. KLAUSHOFER and KOLBER found that there exists a critical value of $1,0$ mg O_2/l in systems which they investigated. At this value the oxygen value is too high for the growth of the anaerobic *Clostridium thermohydrosulfuricum*. However, we have never found *Cl. thermohydrosulfuricum* or any other anaerobic bacterium which has a growth rate high enough to allow a dominating growth in the Swedish Buckau towers.

Disinfectant

A second factor determining the size and activity of the microflora in the extraction is the choice and concentration of disinfectant. The discontinuous addition of disinfectant causes an immediate decrease of the number and activity of the micro-organisms. But after some hours the initial level is reached again, due to the dilution of the disinfectant. Normally HCHO is used as disinfectant in Sweden as elsewhere. In all Swedish sugar factories the Grampositive cocci then dominate according to our investigations. This was valid for all four years of study. But if hydrogen peroxide was used, as in fullscale experiment in one factory, Grampositive, non-spore forming rods will dominate instead. As far as we know there are no reports in the literature about changes in the composition of the microflora after the exchange of disinfectant.

Temperature

The most important factor for microbial growth in the extraction system is undoubtedly the temperature. We have never found any substantial bacterial growth in the extraction system if the temperature exceeded $71^\circ C$.

It is possible to restrict the degradation of sugar by the addition of HCHO or other disinfectants. However, our opinion is that the only way to radically reduce the size and activity of the microflora is to keep the extraction temperature over $71^\circ C$. Unfortunately, this is not always possible for technical reasons, e.g. lack of steam, ineffective heat exchange, low filterability depending on the extraction of polysaccharides from beet. This result coincides with the opinion of HOLLAUS¹² who found that infections with *Bacillus stearothermophilus* can be expected at temperatures between 65 and 70° while the risks of an infection with this species of bacteria is diminished at temperatures above $72^\circ C$.

Dominating bacteria

Many reports have stated that the *Bacillus stearothermophilus*^{5, 6, 17, 18}, *Clostridium thermohydrosulfuricum* and *Cl. thermosaccharolyticum*¹⁹ are the most important infections of the extraction system. But during our studies in the Swedish sugar factories we have never found any spore forming or any anaerobic bacteria to be dominant. Although the spore formers contribute a considerable part (25–40 %) of the total number of organisms, they do not seem to have sufficiently high growth rate to compete with other organisms.

The thermophilic coccus is probably not dominant only in Swedish extraction systems. But the reason why it has never been discovered elsewhere may be due to the fact that it is difficult to cultivate and that it very rapidly loses its viability on laboratory media. It can not be isolated from frozen samples or from samples that have been stored in a refrigerator over night. This indicates that the only way to carry out meaningful investigations on the microflora of sugar extraction is by carrying out the microbial analysis immediately in the factory.

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II

THE COMPOSITION AND BIOCHEMICAL ACTIVITY OF THE THERMOPHILIC MICROFLORA ISOLATED FROM BEET SUGAR EXTRACTION.

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Abstract

The beet sugar extraction contains a large number of different micro-organisms. Of these only a small number is supposed to be important, especially the *Bacillus stearothermophilus* group. In order to determine the composition of the microflora and the properties of the extraction organisms, we isolated and characterized 880 organisms according to morphology, Gram reaction and spore forming ability. Of these 481 were thermophilic organisms biochemically characterized and then divided into six groups. Between 44 and 100 % of the bacteria belonging to these different groups could degrade sucrose. L-lactic acid, acetic acid and ethanol were the most common metabolites of sucrose degradation.

Spore forming organisms constituted 35 % Gram positive non-spore forming rods 37 %, Gram negative rods 21 % and Gram positive cocci 3,5 % of the isolates. Gram positive cocci isolated at 70°C constituted a homogenous group. This group contained the dominant organism tentatively named "*Saccharococcus thermophilus*". All other groups of bacteria were found to be more heterogenous.

Introduction

In the beet sugar extraction there are a great number of different micro-organisms present. These organisms originate from the soil which is transferred into the extraction vessel together with the beets. The majority of these organisms are washed from the cossettes and pass out of the extraction in the raw juice. The remaining bacteria follow the cossettes and meet the extraction juice at an increasing temperature. After a few minutes the temperature has increased to 70–72°C. Some of the thermophilic bacteria show metabolic activity and can grow in the extraction system. If the temperature or the dilution rate decrease, some strains can grow very rapidly and reach high numbers (13). Even though the mesophilic bacteria do not multiply and probably do not have any metabolic activity, some of them also survive the passage through the extraction vessel.

Several authors have isolated and characterized thermophilic bacteria from the beet sugar extraction. Almost all of the isolated organisms were spore forming bacteria. *Bacillus stearothermophilus* has been isolated numerous times from sugar factories e.g. in Austria (15, 17, 18, 31). Germany (1, 11), Great Britain (2, 6), Hungary (34), Japan (25) and USA (32).

Klaushofer and Hollaus (18) described 87 strains isolated from extraction juice. Two-thirds of these were *B. stearothermophilus* strains and one-third was *B. sphaericus*, *B. thermodenitrificans* and *B. coagulans*.

Abdou (1) isolated 68 strains of aerobic, thermophilic bacteria from various stages in the sugar factory process as well as from garden and beet soil. Some bacteria were also isolated from white sugar originating from different countries. All of the isolated micro-organisms were spore forming bacteria, and he assumed that they belonged to the *B. stearothermophilus* group.

Klaushofer and Pollach studied the ability of thermophilic sporeformers to form acids from sucrose and from monosaccharides. They found that different strains are involved and many act preferentially on monosaccharides, while others decompose sucrose more rapidly than its monosaccharide components (20). Klaushofer and Pollach also studied the extracellular formation of invert sugar. However, they found only one species which produced extracellular invertase to any great extent (21).

Klaushofer and co-workers (15, 17, 31) have during several years studied the anaerobic, thermophilic, hydrogen sulphide producing bacteria in extraction towers. They isolated a new species, *Clostridium thermohydrosulphuricum* which could grow at 75°C. This is about ten degrees higher than the maximum temperature for *Cl. thermosaccharolyticum* which also is present in anaerobic sugar extracting systems.

Reports of the presence of asporogenous bacteria in beet sugar extraction are few. Allen *et al* (2) found that the contamination in a diffusion battery was due to thermophilic lactobacilli at the beginning of the battery and later to thermophilic anaerobes.

In an earlier report (13), we have described the distribution, size and activity of the microflora in Swedish beet sugar extraction. We could never observe any spore forming bacteria to dominate. Instead, we have very frequently found a previously undescribed aerobic, heterotrophic, thermophilic Gram-positive coccus (27).

In this paper we describe the morphological and biochemical characterization of 880 of bacterial strains isolated from beet sugar extraction. We have especially emphasized the characterization of the aerobic and facultative anaerobic thermophilic bacteria.

Material and methods

Organisms

All bacteria investigated were isolated from Swedish beet sugar extraction during the campaigns 1977–1980. The bacterial isolates selected for examination were the most frequent types of bacteria and represented a variety of colonial morphologies.

Most of the isolates were stored on agar slants in a refrigerator at 5°C. The thermophilic cocci lost their viability if they not were transferred daily to fresh agar media. However, we found they could also be lyophilized in a solution of skim milk (10 %) and sucrose (2 %).

880 different isolates were characterized according to morphology, Gram reaction and spore forming ability. 299 thermophilic bacteria including 9 actinomycetes were tested at 55°C and 142 at 70°C with respect to their biochemical activities.

Substrates

Tryptone Glucose Extract Agar with sucrose 5 g/l (TS) and Iron Sulphite Agar (ISA) as earlier described (13) were used for determining the composition of the microflora. Agar plates were incubated for 24–48 hours at 35, 55 and 70°C.

The sucrose nutrient solution used for the semiquantitative determination of invertase was composed of tryptone (Oxoid) 5 g/l, Lab Lemco Beef Extract (Oxoid) 3 g/l, K_2HPO_4 1 g/l and sucrose 10 g/l. The same substrate was used for the determination of sucrose metabolites.

Biochemical and morphological tests

Two commercial test kits, API 20 A and 20 B (API System SA, La Balme des Grottes, France), were used. Aerobic bacteria from 16–24 hours old cultures grown on TS agar and anaerobic bacteria from ISA were used as inocula. The test kits were inoculated according to manufacturer's instructions. The morphological tests were performed according to the instructions in the API 20 B test manual. The results were recorded after 48 hours of incubation at 55 or 70°C (3, 26).

Determination of invertase

For a semi-quantitative determination of invertase the bacteria were grown in a sucrose nutrient solution. The tubes were incubated at 55 or 70°C and samples were taken after 5, 10 and 24 hours. For determination of the amounts of fructose and glucose the samples were chromatographed on silica gel plates (Merck Kieselgel 60) with acetone – ethylacetate – water (7:2:1) as the solvent system. The sample volume was one μ l. 0.5 % solutions of sucrose, fructose and glucose were used as standards. The reagent used for developing the plates was aniline-diphenylaniline (12).

For a confirmation of these preliminary screening results, the presence of fructose and glucose was assayed by enzymatic analysis. The test kits were manufactured by Boehringer Mannheim GmbH Diagnostica, Western Germany, catalogue number 139106.

Determination of metabolic products

For the determination of degradation products of sucrose the bacteria were grown in the sucrose broth described. The bacteria were incubated for 72 hours at 55 or 70°C

in test tubes (16×160 mm) with 10 ml substrate. For anaerobic growth, the broth surface in tubes was covered with a 10 mm thick layer of sterile paraffin oil.

Metabolic products were analyzed using gas chromatography as earlier described (13).

Similarity between the isolated bacteria

Based upon the results of 42 different, biochemical, physiological and morphological tests the simple matching coefficients according to Sokal and Michener (33) were calculated.

The 42 tests were the characteristics in Table 1, 2 and 4. In addition to these motility, formation of aggregates and formation of iso-propanol, iso-butanol and acetone were used.

All isolates within each group of bacteria were matched with each other. The results obtained for the two groups of Gram positive cocci are shown in dendrograms (Figures 1 and 2). In Figure 3 the results are summarized for all the thermophilic groups. These diagrams show the percentage of matching pairs at different values in proportion to the total number of pairs in each group.

All computation was done on a Superbrain QD computer (Interic Data System) using a program written by B. Stehn, Swedish Sugar Co. The principle of the computer programme for the cluster analysis was average linkage according to Sokal and Michener (33).

Results

Table 1 gives a general description of the aerobic and facultative anaerobic microflora in the extraction based on the characterization of 840 isolates. More than 90 % of the mesophilic and thermophilic bacteria were rods; of these, most were Gram positive. The spore forming and the non spore forming Gram positive rods constitute about one third each of the total number of those bacteria growing at 35° and 55°C. However, among the bacteria growing at 70°C about 50 % were Gram positive non spore formers and about 25 % spore formers. The number of Gram positive cocci is also the highest at 70°C. Most of these bacteria were strains closely related to the earlier described thermophilic coccus (27). The anaerobic bacteria characterized were all Gram positive spore formers.

We have never observed any fungi in the extraction vessel, but at lower temperatures we have isolated a few strains of yeast as well as some actinomycetes.

TABLE 1. *The composition of the aerobic and facultative anaerobic microflora.*

Temperature °C	Total number of isolates	Gram positive non-spore forming rods	Gram positive spore forming rods	Gram negative rods	Gram positive cocci	Actino-mycetes	Yeast	Fungi
35	263	88 33.5%	98 37.3%	72 27.5%	1 0.3%	1 0.3%	3 1.1%	0 0%
55	428	153 35.7%	165 38.6%	81 18.9%	17 4.0%	12 2.8%	0 0 %	0 0%
70	149	76 51.0%	38 25.5%	23 15.4%	12 8.1%	0 0 %	0 0 %	0 0%
Total	840	317 37.7%	301 35.8%	176 20.9%	30 3.6%	13 1.6%	3 0.4%	0 0%

Metabolic activities

Most of the isolated thermophilic bacteria were characterized according to their metabolic activities (Table 2–5).

TABLE 2. *Aerobes and facultative anaerobes: results of biochemical tests.*

The figures refer to the percentage of isolates within each group of bacteria showing a positive reaction.

Temperature	55°C					70°C			
Test	Gram positive non-spore forming rods n = 101	Gram positive spore forming rods n = 130	Gram negative rods n = 42	Gram positive cocci n = 17	Actino-mycetes n = 9	Gram positive non-spore forming rods n = 69	Gram positive spore forming rods n = 37	Gram negative rods n = 16	Gram positive cocci n = 20
Fructose	95	82	88	94	89	97	97	100	100
Glucose	97	82	93	100	100	100	100	100	100
Galactose	60	57	47	70	44	62	73	56	75
Mannose	60	54	49	70	44	68	70	69	75
Rhamnose	58	55	37	65	11	66	57	50	80
Maltose	79	64	56	82	67	75	84	69	80
Sucrose	93	71	88	100	78	97	97	94	100
Mannitol	65	53	39	76	67	52	54	44	75
Sorbitol	51	61	35	70	44	38	49	25	75
1 (+) arabinose	63	51	49	70	67	66	62	38	75
Starch	40	41	23	47	33	25	35	6	40
Glycerol	60	70	42	70	44	40	49	31	75
Acetoin	8	7	7	0	0	3	0	6	0
H ₂ S	3	6	0	0	0	0	0	0	0
Indole	12	26	5	0	0	3	5	0	0
Nitrite	29	43	23	41	0	51	30	50	40
Gelatine	77	80	72	88	33	68	51	62	80
β-galactosidase	52	60	39	23	0	30	22	13	10
Oxidase	65	57	26	23	67	55	76	56	90
Catalase	63	56	46	76	89	62	96	38	85
Urease	48	25	30	12	0	27	27	44	0
Invertase	6	9	7	18	11	11	3	6	5
Citrate	39	50	19	23	0	30	14	44	10
Fermentation of glucose	29	19	28	12	0	26	8	56	10

Saccharides

Almost all of the aerobic and facultative anaerobic bacteria which were isolated and grown at 70°C could metabolize sucrose, fructose and glucose, while many of the 55°C bacteria, especially those of the Gram positive spore formers, could not. The ability to metabolize other saccharides does not seem to differ markedly between the morphological groups. The main exception is that only one out of 16 strains of Gram negative rods growing at 70°C could degrade starch.

Among the anaerobic bacteria many could not degrade the saccharides tested. This was especially valid for those growing at 55°C.

Nitrite, urease and indol

The reduction of nitrate to nitrite is of special interest as this metabolic activity has been used as a rapid method for detecting bacterial infections in sugar extraction (7,

9). Our results (Table 2) show that 23–51 % of the aerobic and facultative anaerobic isolates could reduce nitrate.

About one third of the isolated bacteria could produce urease. The cocci were exceptions in this character. None of the cocci grown at 70°C and only two of the cocci grown at 55°C could synthesize this enzyme. The production of indol was a relatively common ability among the tested Gram positive rods at 55°C but not at 70°C.

TABLE 3. *Anaerobes: results of biochemical tests.*

The figure refer to the percentage of isolates within each group of bacteria showing a positive reaction.

Temperature	55°C	70°C
Test	Gram positive spore forming rods n = 16	Gram positive spore forming rods n = 24
Glucose	37	75
Mannose	25	33
Rhamnose	6	12
Cellobiose	19	29
Lactose	6	4
Maltose	44	54
Sucrose	44	87
Trehalose	31	33
Melezitose	6	8
Raffinose	31	29
l (+) arabinose	25	25
d (+) xylose	12	42
Mannitol	19	54
Sorbitol	31	21
Glycerol	25	33
Salicin	0	33
Esculin	6	29
Gelatine	75	75
H ₂ S	62	0
Indole	0	4
Catalase	0	0
Invertase	6	8
Urease	62	46

Degradation products from sucrose

The isolated bacteria were tested for their ability to degrade sucrose (Tables 4 and 5). The two most common of the tested degradation products were lactic acid and acetic acid. This was especially valid for the cocci. About one third of the bacteria

TABLE 4. *Number of bacteria producing different amounts of metabolites from sucrose degradation. 55°C isolates.*

The figures refer to the percentage of isolates within each group of bacteria.

Metabolite (mg/l)	Aerobes					Anaerobes
	Gram positive non-spore forming rods n = 101	Gram positive spore forming rods n = 130	Gram negative rods n = 42	Gram positive cocci n = 17	Actino- mycetes n = 9	Gram positive spore forming rods n = 16
Lactic acid						
<5	39	45	40	30	67	100
5-50	38	39	38	12	22	0
50-100	16	10	18	23	11	0
>100	7	6	4	35	0	0
Acetic acid						
<5	33	38	36	7	22	62
5-50	51	56	52	70	56	19
50-100	16	6	12	23	22	19
>100	0	0	0	0	0	0
Propionic acid						
<5	70	69	79	53	100	94
5-50	27	28	21	35	0	0
50-100	3	3	0	12	0	6
>100	0	0	0	0	0	0
Isobutyric acid						
<5	86	79	90	59	78	57
5-50	13	19	10	41	22	12
50-100	1	2	0	0	0	25
>100	0	0	0	0	0	6
Butyric acid						
<5	87	80	90	100	89	100
5-50	11	20	10	0	11	0
50-100	2	0	0	0	0	0
>100	0	0	0	0	0	0
Valeric acid						
<5	90	81	90	82	89	82
5-50	8	19	10	12	11	6
50-100	2	0	0	6	0	6
>100	0	0	0	0	0	6
Ethanol						
<5	63	68	72	88	100	94
5-50	26	24	21	12	0	6
50-100	11	8	7	0	0	0
>100	0	0	0	0	0	0

TABLE 5. *Number of bacteria producing different amounts of metabolites from sucrose degradation. 70°C isolates.*
The figures refer to the percentage of isolates within each group of bacteria.

Metabolite (mg/l)	Aerobes				Anaerobes
	Gram positive non-spore forming rods n = 69	Gram positive spore forming rods n = 37	Gram negative rods n = 16	Gram positive cocci n = 20	Gram positive spore forming rods n = 24
Lactic acid					
<5	32	43	44	15	100
5-50	7	14	0	0	0
50-100	40	27	31	25	0
>100	20	16	25	60	0
Acetic acid					
<5	48	30	44	5	54
5-50	32	48	44	40	21
50-100	20	22	12	45	25
>100	0	0	0	10	0
Propionic acid					
<5	68	65	81	55	92
5-50	23	30	13	45	4
50-100	6	5	0	0	4
>100	0	0	6	0	0
Isobutyric acid					
<5	80	73	100	60	54
5-50	17	24	0	40	21
50-100	3	3	0	0	17
>100	0	0	0	0	8
Butyric acid					
<5	94	84	94	100	100
5-50	6	13	6	0	0
50-100	0	3	0	0	0
>100	0	0	0	0	0
Valeric acid					
<5	95	92	94	95	62
5-50	2	8	6	5	13
50-100	3	0	0	0	17
>100	0	0	0	0	8
Ethanol					
<5	74	70	81	90	83
5-50	22	24	19	10	4
50-100	4	6	0	0	13
>100	0	0	0	0	0

could produce ethanol. However, only some of the bacteria grown at 70°C had this ability.

A striking difference between the aerobes and anaerobes was the total inability of the latter to produce lactic acid from sucrose. The aerobes could not produce iso-propanol, iso-butanol or acetone.

Similarities between the isolated strains

The similarity between the strains of the different morphological groups are shown in Figures 1, 2 and 3. The dendrograms for the thermophilic cocci show that most of the bacteria isolated at 70°C (Figure 1) are closely related but those isolated at 55°C (Figure 2) are more spread in their relationship.

FIGURE 1.



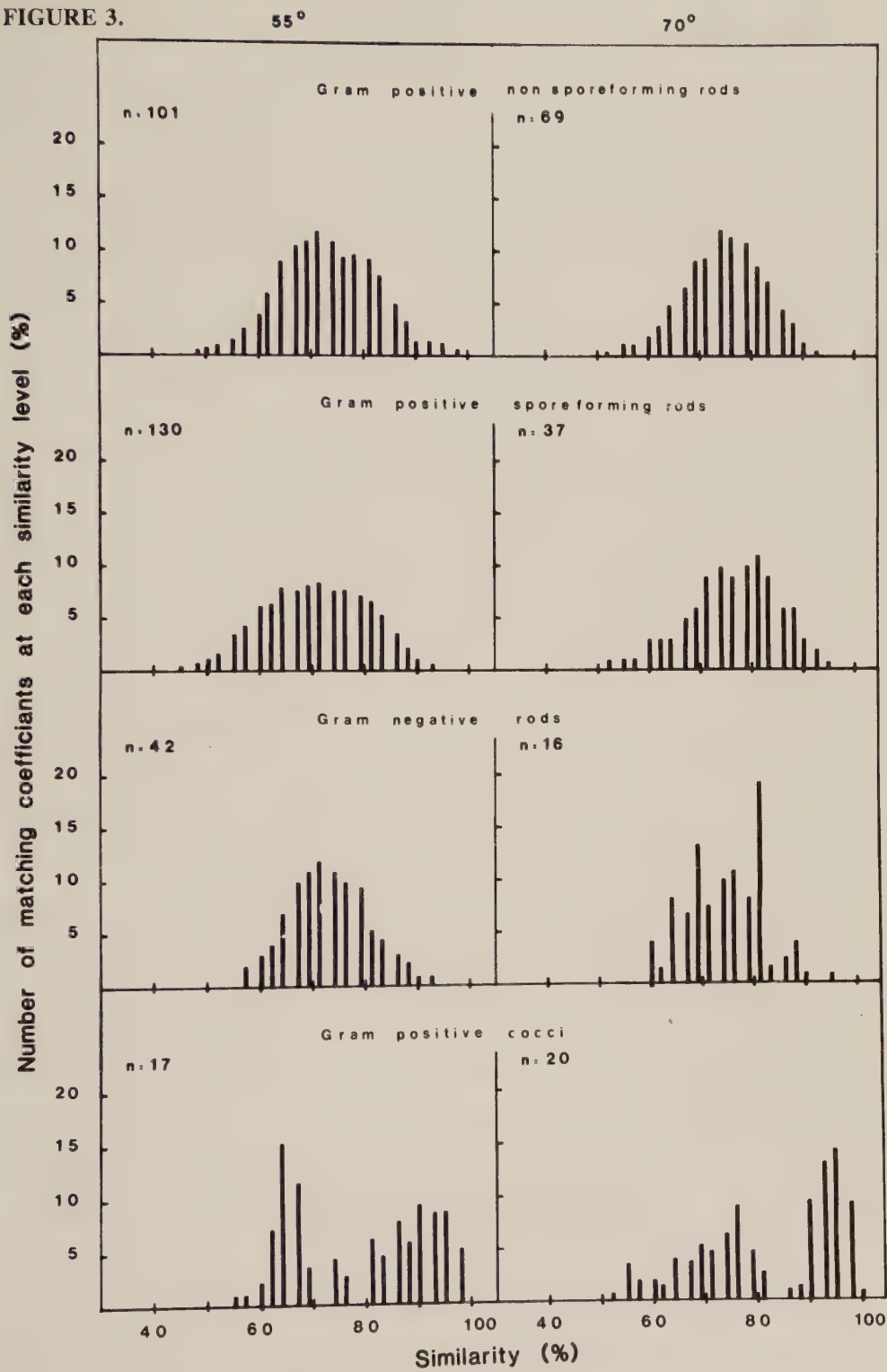
*Dendrogram (multi linkage) of the 70°C Gram positive cocci strains.
The similarity levels are given in percent.*

FIGURE 2



*Dendrogram (multi linkage) of the 55°C Gram positive cocci strains.
The similarity levels are given in percent.*

In Figure 3 the similarities between all the combinations of two bacteria in each group are shown. In the groups of cocci, especially of those isolated at 70°C, there are many pair combinations with matching coefficients exceeding 90 %. This implies a relationship on the level of species. In the other groups, these coefficients normally were considerably lower indicating that most of these bacteria were less closely related. A reason for this can be as the ecological niche become more defined, the heterogeneity decreases, especially for the dominating species.



Similarity of the different groups of isolates.
The similarity within each morphological group of isolates at both 55°C and 70°C.

Discussion

The total microflora present in the beet sugar extraction is large as previously described (13). Of all the quantity and variety of organisms present, only a few species are able to grow and become dominant in the system.

The low number of important organisms is a result of the environmental factors in the system: high temperature (about 70°C), the dilution rate (the system operates continuously), the disinfectant (usually formaldehyde), the redox potential and the nutrients present.

The only genera which have been reported to be important in the beet sugar extraction are *Bacillus*, especially *B. stearothermophilus* (1, 10, 16, 17, 19) and *Clostridium* (14, 15, 19, 23). Recently we described a Gram positive coccus as dominant in Swedish beet sugar extraction (13, 27).

Bacillus sp have been characterized by Abdou (1). The 68 isolates investigated were less active in decomposing different sugars at 65°C than both the 55°C and the 70°C Gram positive spore formers in our investigation. The ability to reduce nitrate to nitrite was positive in 75–80 % of his isolates, but in our investigation only 30–40 % of the Gram positive spore formers were positive in this test.

The characterization of 87 strains of *Bacillus* by Klaushofer and Hollaus (18) showed that most of the strains were active in decomposing different sugars. Acetoin was not formed by their isolates and this was also the case in our investigation.

Abdou characterized all the isolates in his investigation as *B. stearothermophilus*. Klaushofer and Hollaus identified 58 of their 87 strains as *B. stearothermophilus*. In this investigation, we have not made any attempts to identify the bacteria to species level. The study of similarity among the isolates in the six groups showed that they were not closely related except in one group, the 70°C Gram positive cocci. This group contained the Gram positive coccus, tentatively named "*Saccharococcus thermophilus*" (27), which was recorded as dominant species.

Hollaus and Klaushofer (15) studied anaerobic bacteria isolated from beet sugar extraction. All 14 of their isolates produced hydrogen sulphide while in our investigation only 62 % formed hydrogen sulphide at 55°C and none at 70°C.

Although the spore formers constituted 35 % of the isolates, no spore forming bacterium was recorded as dominant species during the four years of our study. This is an interesting observation since all other investigators state that spore formers are the most important organisms.

In our study, Gram positive organisms constituted 80 % of the organisms isolated. When we changed the disinfectant from formaldehyde to hydrogen peroxide (28) the dominant Gram positive coccus was replaced by another Gram positive organism, a *Lactobacillus* sp. Toth-Zsiga (34) investigated raw juices in Hungary and found that 66 % of the isolates were Gram positive organisms. The investigations of Abdou (1), Klaushofer and Hollaus (18) and Hollaus and Klaushofer (15) deal only with Gram positive organisms. These data indicate that the conditions in the beet sugar extraction are more favorable to Gram positive organisms than to Gram negative organisms.

Investigations of other thermophilic environments e.g. hot springs (4, 8) and domestic hot water systems (5) show a wide variety of Gram negative organisms.

which can also be dominant. Gram positive organisms are also present in these environments. An important difference between the beet sugar extraction and these environments is the concentration of nutrients.

The ability of the isolates to degrade sucrose to different metabolites has not been studied in the field of beet sugar microbiology. In our study, we have shown that L-lactic acid, acetic acid and ethanol are not only the most common metabolites but they are also produced in the largest amounts by the isolates. Samples from the beet sugar extraction juice showed the same pattern. That L-lactic acid is the most important metabolite in the beet sugar extraction juice has been shown in many investigations (13, 22, 24, 29, 30). Our study confirms these findings. Winström-Olsen et al (35) performed a study of the metabolites present in the extraction juice. They showed that L-lactic acid, acetic acid and ethanol were the most common metabolites, while butyric acid was not present. The results of the isolates in our study show similar results.

Changes in temperature in the range of 65–74°C did not alter the microflora, nor did minor changes in the dilution rate. When the extraction was stopped for technical reasons and the vessels were emptied, the microflora remained the same when the extraction was restarted. This indicates that the composition of the microflora is stable and if changes can be made, the changes in the environmental parameters must be significant, such as a change in the disinfectant (28).

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III

SACCHAROCOCCUS THERMOPHILUS GEN. NOV., SP. NOV. ISOLATED FROM BEET SUGAR EXTRACTION

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Summary

A Gram-positive, aerobic, heterotrophic, thermophilic, non-spore forming coccus has been found to be the dominating microorganism in Swedish beet sugar extraction plants. Eight strains of this coccus are characterized in terms of their morphology and biochemical reactions.

The main metabolite of sucrose degradation is L-lactic acid. The optimum temperature range is 68–70°C.

Two strains of the coccus were screened for plasmids. One strain contains two plasmids, 38 and 26 megadaltons in size. The other strain contains only one plasmid, 38 megadaltons in size. The characteristics of the coccus differ from other Gram-positive cocci described.

The GC content of the DNA of the coccus and its cell wall composition were found to be similar to those of *Bacillus stearothermophilus*.

It is suggested that the coccus represents a new genus and species, *Saccharococcus thermophilus*. Strain 657 is the type strain and it has been deposited in the Czechoslovak Collection of Microorganisms (Brno) under the number CCM 3586.

Key words: *Saccharococcus thermophilus* – Beet sugar extraction – Thermophile – Aerobic coccus –

Introduction

The juice in the beet-sugar extraction system is a nutritionally rich medium, which contains a number of different micro-organisms (Abdelu, 1970; Haskå and Nystrand, 1982; Hollaus and Klaushofer, 1973; Klaushofer and Parkkinen, 1965; Klaushofer and Hollaus, 1970; Klaushofer and Pollach, 1972). However, as the temperature in the system is about 70°C, only thermophilic micro-organisms can multiply. The micro-organism which then becomes dominant constitutes normally at least 99 % of the total of colony forming units (CFU) which amounts to 10^4 – 10^6 /ml.

The microbial activity in the extraction system is usually evidenced by a drop in the pH, resulting from the production of acids, mainly L(+)-lactic acid (Mauch et al.,

1966; Mc Master and Ravnö 1975; van der Poel, 1975; Schiweck and Büsching, 1972). The activity of the microflora can be suppressed by the addition of a disinfectant, normally formaldehyde (Hollaus and Klaushofer, 1970; Toth-Zsiga, 1967).

The extraction in beet sugar plants in Sweden is carried out in one of two different systems: The Buckau-Wolf tower extraction or the De Danske Sukkerfabrikker (DDS) trough extraction. The former system is more anaerobic than the latter. However, the tower extraction system has a scalding trough, in which the conditions are similar to those of the first part of the DDS trough. In both extractions there is a press water system which operates mainly under aerobic conditions. The extraction system is a continuously operating system with high flow rates (Haskå and Nystrand, 1982). Due to this the retention time of the extracting agent is short, generally 20–30 min (Klaushofer et al., 1971). This means that a micro-organism which is to become dominant in this system must be capable of a generation time in the extraction system of less than 20 min.

The micro-organisms which dominate in beet-sugar extraction have been reported to be species of the genus *Bacillus*, especially the *B. stearothermophilus* group (Abdou, 1970; Klaushofer and Parkkinen, 1965; Klaushofer and Hollaus, 1970) and the genus *Clostridium*, especially *Cl. thermosaccharolyticum* and *Cl. thermohydrosulfuricum* (Hollaus and Klaushofer, 1973; Klaushofer and Pollach, 1972).

Up to now the *B. stearothermophilus* group have been considered to be the only aerobic micro-organisms of importance in beet-sugar extraction. Investigations of Swedish beet-sugar extraction have shown that another type of bacteria dominates, namely an aerobic, Gram-positive, heterotrophic, non-spore-forming coccus (-Haskå and Nystrand, 1982).

In this paper a characterization is given of eight different strains of this coccus. It is suggested that the coccus represent a new genus and species, *Saccharococcus thermophilus*.

Material and Methods

Bacterial strains

The eight strains of the coccus were isolated during beet-sugar extraction in five different sugar factories in Sweden (Table 1). The coccus could only be isolated from fresh samples immediately after removal from the extraction process. The bacteria could be stored for at least five years, if they were freeze-dried in a medium of skim milk (10 %) and sucrose (2 %).

Cultivation

Tryptone Glucose Extract Agar (Oxoid) 24 g/l with an addition of 5 g/l of sucrose was used as substrate (designated TS). Sterilization was performed by autoclaving at 121°C for 20 min. The cultures were incubated at 70°C for 16–24 h. The strains required transfer to fresh medium daily to ensure their viability. For tests performed

at 55°C the organisms were incubated at 55°C for 16–24 h before inoculation of agar plates or tubes.

The growth medium used for gas chromatographic determination of the degradation products of sucrose had the following composition (g/l): tryptone (Oxoid) 5, Lab Lemco Beef Extract (Oxoid) 3, sucrose 10 and K_2HPO_4 1. The substrate was distributed in 16×160 mm test tubes, 10 ml in each, and autoclaved for 20 min at 121°C. The tubes were incubated for 72 h at 55, 60, 65 and 70°C respectively.

TABLE 1. *List of strains*

Strain	Year	Isolated	
		Sugar factory	Process step
508	1977	Örtofta	extraction trough
619	1978	Örtofta	extraction trough
621	1978	Karpalund	extraction trough
656	1979	Hasslarp	extraction trough
657	1979	Jordberga	scalding trough
739	1980	Köpingebro	scalding trough
772	1980	Jordberga	press water system
783	1980	Örtofta	extraction trough

Microscopy

Cultures grown for 16 h on TS agar at 70°C were used. The fixation process used was a modified *Ryter-Kellenberger* fixation (Ryter et al., 1958). The modification was a pre-fixation step with 3 % glutaraldehyde in *Michaelis* acetate-Veronal buffer at pH 6.6 for 1 h. Epon 812 was used as embedding medium using the method of *Luft* (1961) as modified by *Chen Chang* (1973). The observations were made with a Philips EM 300 electron microscope at 60 kW.

Observations with interference phase contrast microscopy were made on cultures grown for sixteen hours on TS agar at 70°C. The cells were suspended in tap water. The microscope used was a Zeiss Forschungsmikroskop Standard WL with interference phase contrast equipment.

Biochemical tests

The biochemical tests were performed with a miniaturized test system: The API-system (API-System, La Balme les Grottes, 38390 Montalieu Vercieu, France).

Five different test strips were used: API 20 B (*Baleux*, 1977), API 20 E (*Nord* et al., 1974), API 50 E (*Logan* et al., 1979), API 50 L (*Dolezil* and *Kirsop*, 1977; *Hofer*, 1976) and API Zym (*Humble* et al., 1977; *Waitkins*, 1977). The strips were inoculated in accordance with the instructions of the manufacturer with one exception. Because the coccus is aerobic the carbohydrate ampoules in the strips API 50 E and API 50 L were not covered with paraffin oil. The strips were incubated at 55°C and 70°C. All the tests were made in duplicate. The results were recorded after 48 h of incubation.

The benzidine test was performed according to *Deibel* and *Evans* (1960).

Analysis of metabolites

Sucrose degradation products were determined by gas chromatography using the method described earlier (*Haskå and Nystrand, 1982*). L-lactic acid and D-lactic acid were determined by enzymatic analysis (*Haskå and Nystrand, 1982*).

Susceptibility to antibiotics and disinfectants

A suspension of cultures grown for 16 h at 55°C and 70°C respectively were seeded on TS agar. After drying the agar plates for 1 h at 55°C, antibiotic discs (ø 6 mm, AB Biodisk, Stockholm, Sweden) were placed aseptically on the agar surface. Incubation was performed at 55°C and 70°C respectively for 24 h and then the inhibition zones were measured. The following antibiotics were used: bacitracin (10 IU/disc), chloramphenicol (30 µg/disc), erythromycin (15 µg/disc), neomycin (30 µg/disc), penicillin-G (500 µg/disc), polymyxin B (10 µg/disc), streptomycin (30 µg/disc) and tetracycline (30 µg/disc).

The susceptibility to disinfectants was tested using an agar plate method (*Andersson, 1970*). TS agar was sterilized by autoclaving for 20 min at 121°C. When the agar was cooled to about 55°C the disinfectant was added and agar plates were poured. This procedure was performed for each concentration of each disinfectant. Immediately after the agar had solidified the plates were inoculated with a loopful of a cell suspension. The cell suspension was made by suspending a culture grown for 16 h at 55°C and 70°C on TS agar in sterile tap water to a density of approximately 10⁸ cells/ml. The plates were incubated at 55°C and 70°C respectively for 24 h and then examined for visible growth. The disinfectants listed in Table 2 were used.

TABLE 2. *Data for the eight disinfectants*

Disinfectant	Active substance	% (v/v)	Manufacturer
Formaldehyde	Formaldehyde	35.0	Perstorp AB, Perstorp, Sweden
Hydrogen peroxide	Hydrogen peroxide	35.0	EKA AB, Surte, Sweden
Ekarox B 1	Hydrogen peroxide	34.5	EKA AB, Surte, Sweden
	Ethaneperoxoic acid	0.5	
Ekarox B 5	Hydrogen peroxide	32.5	EKA AB, Surte, Sweden
	Ethaneperoxoic acid	2.5	
Ekarox B 10	Hydrogen peroxide	30.0	EKA AB, Surte, Sweden
	Ethaneperoxoic acid	5.0	
Busan 881	Disodiumcyanodithiocarbamate	14.7	Buckman Laboratories SA, Gent, Belgium
	Potassium N-methyldithiocarbamate	20.3	
Antiformin DMT	Dimethyldithiocarbamate	42.0	Stockhausen & Cie, Krefeld, West Germany
Nalco 247	Disodiummethylenedisithiocarbamate	9.0	Nalco Chemical Co, Oak Brook, Illinois, USA
	Sodiumdimethyldithiocarbamate	14.0	
	Ethylenediamine	10.0	

DNA base composition

The DNA was diluted in 0.1 SSC (SSC=0.15 M sodiumchloride+0.015 M trisodiumcitrate) for T_m determination (T_m=the temperature at the midpoint of the thermal melting curve).

The T_m determination was performed with a Gilford 250 instrument equipped with a thermoprogrammer type 2527. As a standard the DNA prepared from *E. coli*

B NCTC 10537 with a GC % = 52 was used (GC = guanine + cytosine). The amount of GC of the DNA was calculated to *Mandel et al.* (1979).

Plasmid screening

The methods used by *Ståhl* and *Bernander* (1983) were used to determine the presence of plasmids.

Cell wall chemistry

The methods of *Schleifer* and *Kandler* (1972) were used.

Similarity between the strains

The similarity between the strains was calculated as matching coefficients according to *Sokal* and *Michener* (1958).

Results

Colonial morphology

The colonies on TS agar were circular with a diameter of 1–2 mm. The surface was dull and smooth or slightly rough. The edge was regular and the colonies were white to yellow brown and opaque.

Cellular morphology

The morphology of the bacterium is seen in Figs. 1 and 2. It is a coccus which grows in irregular clusters (Fig. 1). The size of the single cells is 1–2 μm in diameter (Fig. 2). The clusters seen in Fig. 1 have a diameter of 5–15 μm . Consequently one colony forming unit (CFU) can consist of 50–100 cells. In Fig. 2 the fine structure of the cells is seen in an electron micrograph. The cell wall organization is similar to that of Gram-positive bacteria. The thick murein layer is clearly visible as is the cell membrane. The cell wall is covered with a thin slime layer which possibly causes the strong adhesion of the cells to each other. The cell membrane has invaginations which are probably mesosomes. The coccus is non motile and lacks flagella. No pili or other surface structures can be observed. The cells divide in more than one plane to form the irregular clusters.

Susceptibility to antibiotics and disinfectants

The antibiograms for the eight strains tested were quite similar. The strains were resistant to polymyxin B and sensitive to all other antibiotics tested: chloramphenicol, erythromycin, neomycin, penicillin-G, streptomycin, tetracycline and bacitracin.

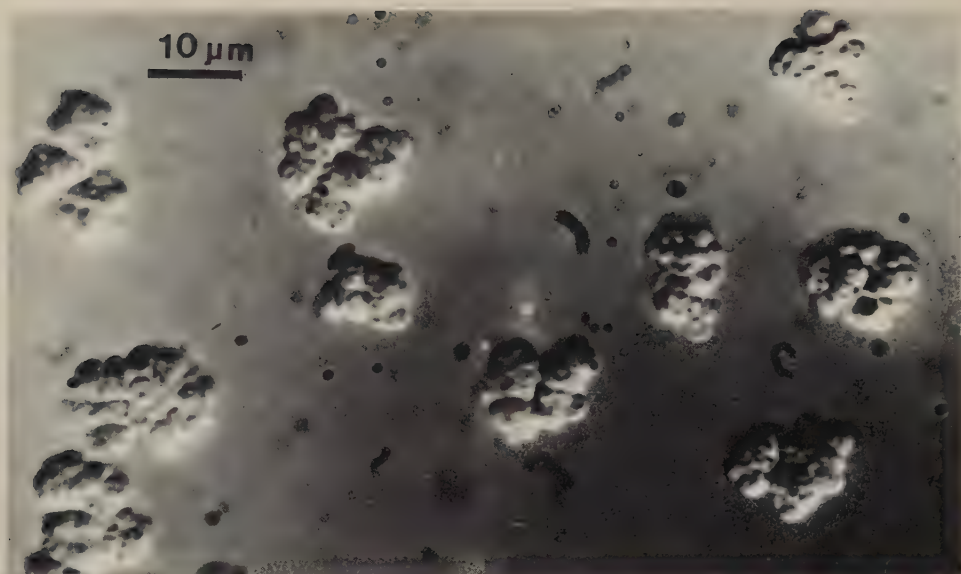


Fig. 1. Interference phase constast micrograph (Normarski) of clusters of cells suspended in tap water. the bar equals 10 μm.

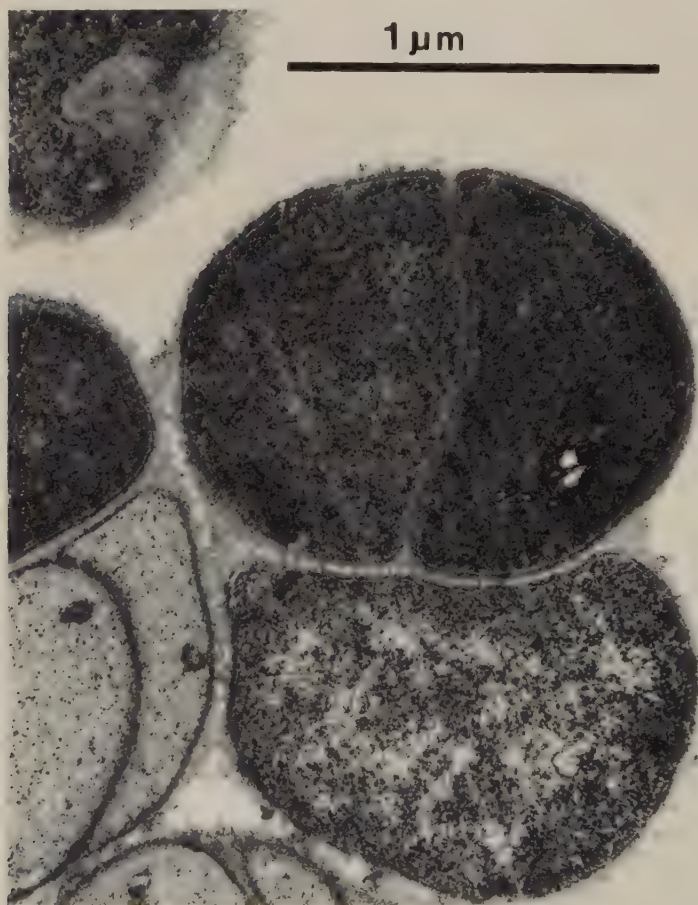


Fig. 2. Electron micrograph of the cocci. The bar equals 1 μm.

The results in Table 3 show that the eight strains were relatively tolerant to formaldehyde and were sensitive to Busan 881, Antiformin DMT, Nalco 247, hydrogen peroxide and to the three Ekarox preparations B 1, B 5 and B 10.

TABLE 3. *Sensitivity to disinfectants*

Disinfectant	Inhibiting concentration (mg/l)
Formaldehyde	50-75
Hydrogen peroxide	20
Ekarox B 1	10-20
Ekarox B 5	10
Ekarox B 10	5-10
Busan 881	5-10
Antiformin DMT	5-20
Nalco 247	10-30

All figures of concentration refer to the total amount of active substances in the disinfectants. For disinfectants with more than one active substance, the amounts of the different active substances are given in Table 2.

Biochemical tests

The results of the biochemical tests are shown in Table 4 and Table 6. There were only small differences in biochemical activity in the eight strains tested at the two temperatures 55°C and 70°C. Of totally 8×80 biochemical test results for the eight strains only 23 results differed at either of the two temperatures. Twenty of these were carbohydrate degradations. As far as the carbohydrates tested are concerned the eight strains were most active in decomposing mono- and disaccharides. No sugar alcohols were degraded at 55°C but some at 70°C.

Three strains reduced nitrate to nitrite at both temperatures. Gelatine and tributyrine were degraded by all strains at both temperatures. All strains produced the same enzymes at both temperatures with the exception of strain 508, which was the only strain to produce trypsin at 55°C.

Metabolites of sucrose degradation

All strains produced lactic acid as the main metabolite of sucrose degradation at 55, 60, 65 and 70°C. Enzymatic analysis showed that only the L-form is produced. None of the strains produced any D-lactic acid. The amounts of the different metabolites produced at 70°C are shown in Table 5. At 65°C the production of metabolites was the same as at 70°C but at 55 and 60°C the final concentration was lower.

Similarity between the strains

In Fig. 3 the similarity between the eight strains is shown in a dendrogram. The similarity was found to be 90-97% based on 103 characteristics.

TABLE 4. *Biochemical tests*

Test	Organism								API System
	508 70°C	619 70°C	621 70°C	656 70°C	657 70°C	739 70°C	772 70°C	783 70°C	
<i>Mono-, Di- and Tri-</i>									
<i>saccharides</i>									
<i>Mono</i>									
Galactose	+	+	+	+	+	+	+	+	20B, 50E, 50L
Glucose	+	+	+	+	+	+	+	+	20B, 20E, 50E, 50L
d-levulose	+	+	+	+	+	+	+	+	50E, 50L, 20B
d+mannose	+	+	+	+	+	+	+	+	20B, 50E, 50L
Rhamnose	+	+	+	+	+	+	+	+	20B, 20E, 50E, 50L
l-sorbose	+	+	+	+	+	+	+	+	50E, 50L
<i>Di</i>									
d+cellobiose	+	+	+	+	+	+	+	-	50E, 50L
Lactose	-	+	+	-	-	+	-	-	50E, 50L
Maltose	+	+	+	+	+	+	+	+	20B, 50E, 50L
d+melibiose	+	+	+	+	+	+	+	+	50E, 50L, 20E
Saccharose	+	+	+	+	+	+	+	+	20B, 20E, 50E, 50L
<i>Tri</i>									
d+melizitose	-	-	-	-	-	-	-	-	50E, 50L
d+raffinose	-	-	-	-	-	-	-	-	50E, 50L
<i>Pentoses</i>									
d-arabinose	+	+	+	+	+	+	+	+	50E, 50L
l+arabinose	+	+	+	+	+	+	+	+	20B, 20E, 50E, 50L
Ribose	+	+	+	+	+	+	+	+	50E, 50L
d+xylose	+	+	+	+	+	+	+	+	50E, 50L
l-xylose	+	+	+	+	+	+	+	+	50E, 50L
<i>Sugar alcohols</i>									
Adonitol	-	-	-	-	-	-	-	-	50E, 50L
Dulcitol	-	-	-	-	-	-	-	-	50E, 50L
Erythritol	-	-	-	-	-	-	-	-	50E, 50L
meso-inositol	-	-	-	-	-	-	-	-	50E, 50L, 20E
Mannitol	+	+	+	+	+	+	+	+	20B, 20E, 50E, 50L
Sorbitol	+	+	+	+	+	+	+	+	20B, 20E, 50E, 50L
<i>MISC compounds</i>									
Acetate	-	-	-	-	-	-	-	-	50E
n-acetylglucoseamine	+	+	+	+	+	+	+	+	50E, 50L
Amygdalin	-	-	-	-	-	-	-	-	20E, 50E, 50L
Amylose	-	-	+	-	-	+	-	-	50E, 50L
Arbutin	+	-	-	-	-	-	-	-	50E, 50L
Citrate	-	-	-	-	-	-	-	-	20B, 20E, 50E
Esculin	+	-	+	+	-	-	+	+	50E, 50L
Gluconate	-	+	-	-	-	-	-	-	50E, 50L
methyl-d-glucoside	-	-	-	-	-	-	-	-	50E, 50L
Glycerol	+	+	+	+	+	+	+	+	20B, 50E, 50L
Malonate	-	-	-	-	-	-	-	-	50E
methyl-d-mannoside	-	-	-	-	-	-	-	-	50E, 50L
Mucate	-	-	-	-	-	-	-	-	50E
Salicin	-	-	-	-	-	-	-	-	50E, 50L
methyl-xyloside	-	-	-	-	-	-	-	-	50E, 50L

TABLE 4 (cont.) *Biochemical tests*

Test	Organism					API System		
	508 70°C	619 70°C	621 70°C	656 70°C	657 70°C	739 70°C	772 70°C	783 70°C
<i>Enzymes</i>								
Chymotrypsin	+	+	+	+	+	+	+	Zym
Esterase	+	+	+	+	+	+	+	Zym
Esterase lipase	+	+	+	+	+	+	+	Zym
α -fucosidase	-	-	-	-	-	-	-	Zym
α -galactosidase	+	+	+	+	+	+	+	Zym
β -galactosidase	-	-	-	-	-	-	-	Zym
β -glucuronidase	-	-	-	-	-	-	-	Zym
N-acetyl- β -glucos- aminidase	-	-	-	-	-	-	-	Zym
α -glucosidase	+	+	+	+	+	+	+	Zym
β -glucosidase	-	-	-	-	-	-	-	Zym
α -mannosidase	-	-	-	-	-	-	-	Zym
Phosphatase, acid	+	+	+	+	+	+	+	Zym
Phosphatase, alkaline	+	+	+	+	+	+	+	Zym
Phosphoamidase	+	+	+	+	+	+	+	Zym
Trypsine	-	-	-	-	-	-	-	Zym
Catalase	+	+	+	+	+	+	+	20B, 50L
Orto-nitro-phenyl- galactosidase	-	-	-	-	-	-	-	20B, 20E, 50L
Oxidase	+	+	+	+	+	+	+	20B, 20E
Tetrathionate reductase	+	+	+	+	+	+	+	50E
Urease	-	-	-	-	-	-	-	20B, 20E, 50L
<i>Amino acid reactions</i>								
Arginine dehydrolase	-	-	-	-	-	-	-	20E, 50L
Cystine arylamidase	-	-	-	-	-	-	-	Zym
Indol	-	-	-	-	-	-	-	20B, 20E
Leucine arylamidase	+	+	+	+	+	+	+	Zym
Lysine decarboxylase	-	-	-	-	-	-	-	20E
Ornithine decarboxylase	-	-	-	-	-	-	-	20E
Tryptophane deaminase	-	-	-	-	-	-	-	20E
Valine arylamidase	-	-	-	-	-	-	-	Zym
<i>Macromolecules</i>								
Dextrin	-	-	+	-	-	+	-	50E, 50L
DNA-se	-	-	-	-	-	-	-	50E
Gelatine	+	+	-	+	+	+	+	20B, 20E
Glycogen	-	-	-	-	-	-	-	50E, 50L
Inulin	-	-	-	-	-	-	-	50E, 50L
Lipase	+	+	+	+	+	+	+	50E
Pectate	+	+	-	-	-	-	-	50E
Starch	-	-	-	-	-	-	-	20B, 50E, 50L
<i>MISC reactions</i>								
H ₂ S	-	-	-	-	-	-	-	20B, 20E
Methyl red	+	+	+	+	+	+	+	50E
Nitrite	-	-	+	-	+	-	-	20B, 20E, 50L
N ₂ gas	-	-	-	-	-	-	-	20B, 20E, 50L
VP	-	-	-	-	-	-	-	20B, 20E, 50L

TABLE 5. *Metabolites of sucrose degradation at 70 °C*

Strain	Metabolite	L(+)-lactic acid mg/l	Acetic acid mg/l	Propionic acid mg/l	Iso-butyric acid mg/l
508		350	70	30	20
619		200	200	5	5
621		250	60	5	30
656		500	25	5	20
657		500	40	5	20
739		200	70	20	40
772		300	30	20	5
783		400	30	20	5

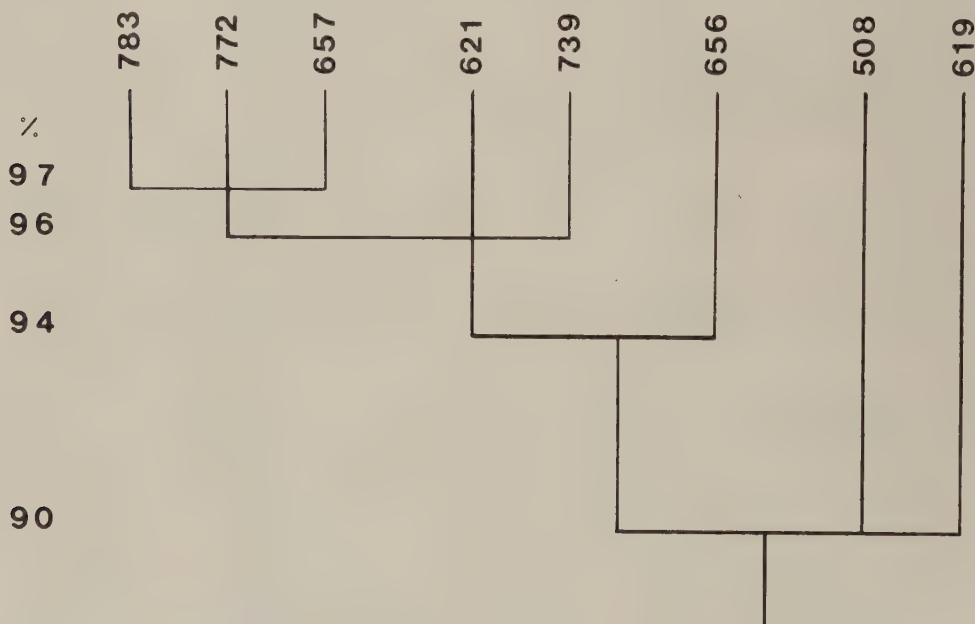


Fig. 3. Dendrogram (multi-linkage) of the similarity in the eight strains.

Plasmid screening

Two strains were screened for plasmids. The presence of plasmids was demonstrated and the result is given in Fig. 4.

Cell wall chemistry

Partial acid hydrolysis of the cell wall showed the following ratio of amino acids: D-Glutamic acid: meso-diaminopimelic acid (meso-DAP):D-Alanine=1:1:1.7. This means that the coccus contains a peptidoglycan type which is directly crosslinked and with meso-DAP as diamino acid. According to the classification system of *Schleifer and Kandler (1972)* is the peptidoglycan of the A 1 γ type. No glycerol or

ribitol could be detected by gas chromatographic analysis which indicates that no teichoic acids are present.

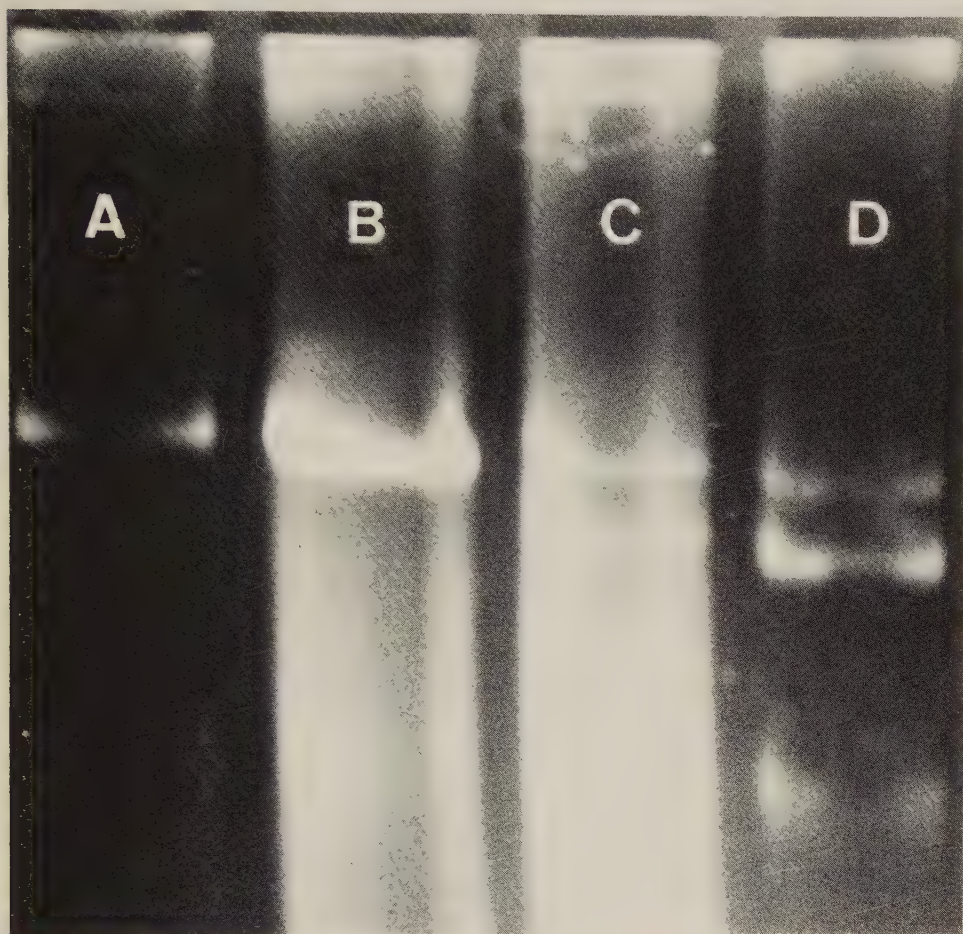


Fig. 4. Agarose gel showing plasmids in two strains of *Saccharococcus thermophilus* and two strains of *Bacillus stearothermophilus*.

Lane A: *B. stearothermophilus* 54 SC: pSE 407 38 megadaltons (mdal).

Lane B: *S. thermophilus* strain 621: one plasmid 38 Mdal.

Lane C: *S. thermophilus* strain 656: two plasmids 38 Mdal and 26 Mdal.

Lane D: *B. stearothermophilus* ATCC 7954: pSE 407 38 Mdal and pSE 409 26 Mdal.

Discussion

The coccus described in this paper has been found to be the most troublesome micro-organism in the Swedish sugar industry (*Haskå and Nystrand, 1982*). It causes losses of sucrose and is therefore of economic interest. For this reason it is important to have a thorough knowledge of the organism's properties.

The source of the coccus is the soil which enters the beet-sugar extraction system together with the beets. Obviously the coccus is wide-spread since it has been found

in five different sugar factories during four campaigns. It is a well documented fact that vegetative cells of thermophilic micro-organisms can be isolated from soil where the temperature never or seldom reaches the thermophilic range (Allen, 1953; Castenholz, 1979).

The properties of this coccus, in particular its optimum growth temperature, its characteristic growth rate and its formaldehyde resistance, makes it suitable for growth in beet-sugar extraction systems.

Descriptions of thermophilic organisms are not very common since there are few environments that have such a high temperature that thermophilic micro-organisms will grow. In the beet-sugar extraction system there is an ecological niche where the coccus described can grow and become important. Other thermophilic environments which have been reported are hot springs (Brock, 1978; Tansey and Brock, 1978) and domestic hot water heaters (Brock and Boylen, 1973), but none of these systems operate continuously as does the beet-sugar extraction system.

L(+)-lactic acid is the main metabolite of sucrose degradation for a number of micro-organisms active during beet sugar extraction (McMaster and Ravnö, 1975; van der Poel, 1975; Shiweck and Büsching, 1972) and analysis of L(+)-lactic acid is one of the methods used for the detection of microbial activity (Mauch et al., 1966; van der Poel, 1975; Shiweck and Büsching, 1972). In this respect our coccus does not differ from other micro-organisms which have been reported to dominate in the extraction systems (Hollaus and Klaushofer, 1973; Klaushofer and Parkkinen, 1965; Klaushofer and Hollaus, 1970; Klaushofer and Pollach, 1972).

Klaushofer et al., (1978) have determined the amounts of L-lactic acid and D-lactic acid produced at different temperatures by the mixed microflora in beet-

TABLE 6. Comparison of "*Saccharococcus thermophilus*" with phenetically similar bacteria

Test	" <i>Saccharococcus thermophilus</i> "	<i>Pediococcus acidilactici</i> ^a	<i>Streptococcus thermophilus</i> ^b	<i>Planococcus</i> ^c	<i>Bacillus stearothermophilus</i> ^d
Cell arrangement	Spheres in irregular clusters	Spheres in pairs or tetrads	Spheres in pairs or long chains	Spheres, singly, pair, threes or tetrads	Rods
Catalase	+	—	—	+	+
Benzidine test	+	—	—	+	ND
Optimum temperature °C	68–70	40	40–45	20–37	50–65
Growth at 50 °C	+	+	+	ND	+
Lactic acid	L	DL	D	ND	L
Sucrose	+	—	+	—	+
Lactose	—	+	+	—	+
Mannitol	+	—	—	+	+
Sorbitol	+	—	ND	ND	—
Xylose	+	+	—	ND	+
Salicin	—	+	—	ND	—
VP reaction (acetoin)	—	+	—	—	—
Gelatine hydrolysis	+	—	—	+	+
Arginine hydrolysis	—	+	—	—	ND
Oxidase	+	ND	ND	—	+
Phosphatase	+	ND	ND	—	+
Lipase	+	ND	ND	—	+
Cell wall peptidoglycan	direct cross-linked meso-DAP	L-Lys-D-Asp	L-Lys-L-Ala ²⁻³	L-Lys-D-Glu or meso-DAP	direct cross-linked meso-DAP
GC content in the DNA (mol%)	47.8 ± 0.2	44	34–46*	39–52	49–53

a, b Data from Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974; Sharpe, 1979).

c, d Data from Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974).

* = this is the range for the genus *Streptococcus*. The data on GC content of *S. thermophilus* is not known. ND = not determined.

sugar raw juice. They found that the amount of D-lactic acid decreased when the temperature was increased and above 70°C all the lactic acid produced was of the L-form. Similar results were obtained by Mauch et al. (1966) using extracts of sugar beets and a mixed microflora. The coccus in this study did not produce any D-lactic acid at the temperatures tested.

According to Bergey's Manual of Determinative Bacteriology 8th edition (Buchanan and Gibbons, 1974) there are no groups of micro-organisms which correspond to our coccus. Thermophilic species are described in some genera which are otherwise dominated by mesophilic organisms, e.g. *Bacillus*, *Clostridium*, *Lactobacillus*, *Thiobacillus* and *Nitrosomonas* (Brock, 1978; Castenholz, 1979), but no aerobic, heterotrophic, thermophilic, Gram-positive cocci are described. Reference literature on the taxonomy of aerobic, heterotrophic, thermophilic, Gram-positive cocci is non-existent.

In Table 6 morphological and physiological characteristics of the coccus are compared to other phenetically similar bacteria. The temperature characteristics of *Saccharococcus thermophilus* are different from those of all other Gram-positive cocci described. The only other Gram-positive cocci described which have meso-diamino pimelic acid as diamino acid in the peptidoglycan are *Mircococcus cryophilus* (Buchanan and Gibbons, 1974) and *Planococcus halophilus* (Novitsky and Kushner, 1976). The GC content in the DNA of *Saccharococcus thermophilus* is within the range of the genus *Planococcus*, but the planococci are motile and have a different cell arrangement.

Saccharococcus thermophilus has the same type of peptidoglycan as is found in *Bacillus stearothermophilus* (Forrester and Wicken, 1966; Schleifer and Kandler, 1972; Sutow and Welker, 1967). No cell wall teichoic acids have been demonstrated in *Saccharococcus thermophilus*, while cell wall teichoic acids are reported to be present (Forrester and Wicken, 1966) or absent (Sutow and Welker, 1967) in *Bacillus stearothermophilus*. The physiological and biochemical characteristics of *Saccharococcus thermophilus* do not differ from the known characteristics of *Bacillus stearothermophilus*, but the morphology is different (Table 6) (Klaushofer and Hollaus, 1970).

The presence of plasmids is interesting, especially as the plasmids demonstrated are of the same size as those found in *Bacillus stearothermophilus*. However, the role of plasmids in taxonomy is not clear.

On the basis of the results presented in this paper it is suggested that the coccus represent a new genus and species.

The description of *Saccharococcus thermophilus* is as follows.

Description of Saccharococcus gen. nov.

Sac. cha. ro. coc'cus. Gr. N. *sacchar* sugar; L. n. *coccus* a grain, berry; M. L. n. *saccharococcus* the sugar coccus, a coccus isolated from beet sugar extraction.

Spheric cells of 1–2 μm in diameter occurring in irregular clusters. Non motile. No endospores formed.

Gram-positive. The cell wall contains peptidoglycan with meso-diaminopimelic acid as diamino acid. Teichoic acids are not present.

The cells are lysed by 100 $\mu\text{g/ml}$ of egg white lysozym.

Aerobic and heterotrophic. Catalase and oxidase are produced. Thermophilic with an optimum temperature range of 68–70°C and a maximum temperature range of 75–78°C.

Acid but no gas is produced from most mono- and disaccharides. L(+)-lactic acid is the main metabolite by carbohydrate degradation.

Description of type species Saccharococcus thermophilus sp. nov.

Ther.mo'phi.lus. Gr. n. *therme* heat; Gr. adj. *philus* loving; M.L. adj. *thermophilus* heat-loving.

The various biochemical characteristics are given in Table 4. Otherwise the description is as for the genus.

Isolated in Swedish beet-sugar extraction at 70°C where the redox potential is more than – 300 mV.

The GC content of the DNA is 47.8 ± 0.2 mol %. Some strains contains plasmids, 38 and 26 megadaltons in size.

Strain 657 is suggested as the type strain. The characteristics are given in Table 4.

The type strain is deposited in the Czechoslovak Collection of Microorganisms (Brno) under number CCM 3586.

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IV

DISINFECTANTS IN BEET SUGAR EXTRACTION.

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Abstract

In a laboratory investigation the inhibiting activity of eight disinfectants were tested against 220 aerobic and facultative anaerobic thermophilic bacteria isolated from beet sugar extractions.

The two most important bacteria found in the beet sugar extraction, a Gram positive coccus tentatively named *Saccharococcus thermophilus* and a thermophilic *Lactobacillus* sp, were studied in more detail.

The aim of this study was to find an alternative disinfectant system to formaldehyde.

The following disinfectants were used: formaldehyde, hydrogen peroxide, Ekarox B 1, Ekarox B 5, Ekarox B 10 (Ekarox preparations are different mixture of hydrogen peroxide and ethane peroxy acid) and three dithiocarbamates Nalco 247, Busan 881 and Antiformin DMT.

Five of the disinfectants were also tested in different combinations in full scale experiments in beet sugar extraction. The changes in the microflora as well as in the production of L-lactic acid were recorded. The temperature was carefully monitored.

The results obtained in the laboratory study were in agreement with the results obtained in the full scale experiments

Ekarox B 10 or Ekarox B 10 in combination with Busan 881 were found to be valuable and effective alternatives to formaldehyde, but it was very important to have a sufficient high temperature.

Introduction

In the sugar industry there is a need to use disinfectants in the extraction step of the process to prevent losses of sucrose, undesirable decrease in pH and formation of metabolites, all caused by micro-organisms.

The disinfectant normally used since the beginning of the sugar industry has been formaldehyde (HCHO). Some of the advantages of the use of formaldehyde are its good antimicrobial activity and its degradation in the further sugar processing into harmless substances (7). However, formaldehyde has disadvantages too, especially

for those who handle it. Formaldehyde is reported to be allergenic (5, 6, 12, 20, 26) and carcinogenic (29), facts which makes it necessary to set very low limit values (3).

Efforts have been made to find alternatives to formaldehyde in the sugar industry. During the last decades many different compounds have been tested i.e. quaternary ammonium compounds (10, 17, 18, 19, 33), iodide compounds (14, 15, 18, 34, 35), sulphur dioxide (23) and esters of p-hydroxybenzoic acid (4). None of these compounds are in use as disinfectants in the system of beet sugar extraction.

A group of chemicals with a strong antimicrobial effect is the dithiocarbamates of which some have been tested in the sugar industry (2, 8, 11, 13, 24, 30).

Recently hydrogen peroxide has also been tested (35). Hydrogen peroxide is advantageous in regards to its harmless decomposition products, water and oxygen.

Ethane peroxoic acid is a compound which has a very strong antimicrobial effect (28). In this study it has been used in mixtures with hydrogen peroxide in the disinfectants Ekarox B 1, B 5 and B 10.

There are a large number of different bacteria present in the beet sugar extraction (16). It is not known in advance which bacteria will be dominant when a change in the conditions of the environment, such as a change of disinfectant, is made. For this reason, a large number of thermophilic bacteria were isolated from beet sugar extraction in order to use them in a comparative laboratory study of eight disinfectants.

In addition to the comparative laboratory study, D values were determined by survival experiments using the most important bacteria for the different disinfectants. The D value is the time required to kill 90% of the organisms.

The intension of the laboratory study was to compare the disinfectants with respect to their ability to inhibit a broad spectrum of thermophilic bacteria under laboratory conditions.

Five of the disinfectants in different combinations were also tested in full scale experiments in sugar factories. The intension of these full scale experiments was to determine the feasibility of using an alternative disinfectant system of formaldehyde.

The following disinfectants were tested in laboratory experiments: formaldehyde, hydrogen peroxide, Ekarox B 1, Ekarox B 5, Ekarox B 10, Nalco 247, Antiformin DMT and Busan 881. Five of these were tested in different combinations in full scale experiments: formaldehyde, hydrogen peroxide, Ekarox B 10, Antiformin DMT and Busan 881.

Material and methods

Organisms

Two hundred and twenty organisms were tested. The organisms were a representative selection of thermophilic bacteria found in beet sugar extraction.

All the strains had an optimum growth temperature range of 55–70°C but were able to grow at 55°C.

The strains were divided into the following groups:

Gram positive spore forming rods	106 strains
Gram positive non-spore forming rods	67 strains
Gram negative rods	30 strains
Gram positive cocci	11 strains
Actinomycetes	6 strains

All the strains were aerobic or facultative anaerobic.

The test organisms were taken from a culture collection of 880 organisms isolated from beet sugar extraction. The distribution of the test organisms in the different groups described above were the same as in the culture collection. All recorded dominating strains were included. The test organisms could be regarded as potential successors to the present dominating organisms.

The Gram positive cocci, which dominated in the extraction, were a homogenous group in comparison to the other groups and consisted mainly of *Saccharococcus thermophilus* strains (22).

Two species of dominating organisms were used in the survival experiments, a Gram positive coccus *Saccharococcus thermophilus* strain 657 and a Gram positive non-spore forming rod, a thermophilic *Lactobacillus* sp strain 771.

Substrates

The agar substrate used was Tryptone Glucose Extract Agar (Oxoid) 24 g/l with addition of sucrose 5 g/l. The pH was 6.8 ± 0.2 . The substrate was designated TS.

In the survival experiments a broth with the following composition was used: Tryptone (Oxoid) 5 g/l, Lab Lemco Beef Extract (Oxoid) 3 g/l, sucrose 5 g/l and K_2HPO_4 1 g/l. The pH was adjusted to 6.8 ± 0.2 . The substrate was autoclaved in one liter portions for 20 minutes at $121^\circ C$. The substrate was designated TSB.

Disinfectants

The disinfectants listed in Table 1 were used.

All figures of concentration in the text refer to the amounts of active substances.

TABLE 1. *Data for the eight disinfectants*

Disinfectant	Active substance	% (v/v)	Manufacturer
Formaldehyde	Formaldehyde	35.0	Perstorp AB, Perstorp, Sweden
Hydrogen peroxide	Hydrogen peroxide	35.0	EKA AB, Surte, Sweden
Ekarox B1	Hydrogen peroxide	34.5	EKA AB, Surte, Sweden
	Ethaneperoxoic acid	0.5	
Ekarox B5	Hydrogen peroxide	32.5	EKA AB, Surte, Sweden
	Ethaneperoxoic acid	2.5	
Ekarox B10	Hydrogen peroxide	30.0	EKA AB, Surte, Sweden
	Ethaneperoxoic acid	5.0	
Busan 881	Disodiumcyanodithiocarbamate	14.7	Buckman Laboratories SA, Gent Belgium
	Potassium N-methyldithiocarbamate	20.3	
Antiformin DMT	Dimethyldithiocarbamate	42.0	Stockhausen & Cie, Krefeld, West Germany
Nalco 247	Disodiummethylenedisithiocarbamate	9.0	Nalco Chemical Co, Oak Brook, Illinois, USA
	Sodiumdimethyldithiocarbamate	14.0	
	Ethylenediamine	10.0	

Agar plate test method

The TS substrate was autoclaved (20 minutes, 121°C). After cooling to 50°C the disinfectants were added and mixed with the substrate. Before adding the disinfectant, the volume of the substrate was adjusted to avoid dilution by the addition of the disinfectant. One batch of substrate was made for each concentration of each disinfectant (1).

For disinfectants with more than one active component, the figure of ppm refers to the total amount of active substances e.g. Ekarox B 10 (hydrogen peroxide 30.0 %, ethaneperoxoic acid 5.0 %) in a concentration of 50 ppm means that there are 50 ppm of Ekarox B 10 active substances in the substrate which are 42.8 ppm of hydrogen peroxide and 7.2 ppm of ethaneperoxoic acid.

Agar plates were prepared and allowed to solidify. The plates were then inoculated with a loop of cell suspensions containing approximately 1×10^8 cells/ml. Plates were incubated at 55°C and were examined for visible growth at 48 hours.

Survival experiments

The survival experiments were performed in 2 litre vessels containing one litre of TSB. The vessels were inoculated with one ml of a cell suspension containing approximately 1×10^8 cells/ml. The cell suspensions were obtained by harvesting cells grown on TS agar plates at 70°C for 16 hours and suspending them in sterile tap water. After addition of disinfectant the vessels were stirred and samples were removed every 10 minutes for at least two hours (27). Sodium thiosulfate 0.1 % was used as neutralizer according to Skaily et al (27). The initial concentration of organisms was about 1×10^5 CFU/ml. The temperature was 70°C. The calculations of disinfectant concentrations were the same as given for the agar plate test method.

The samples were diluted (27) and spread on TS agar plates which were incubated 24 hours at 70°C.

The results were used to calculate the D values (Table 3) for two organisms, *Saccharococcus thermophilus* strain 657 and a thermophilic *Lactobacillus* sp strain 771.

Disinfectants in full scale experiments

The disinfectants were added both in the extraction trough and in the press water system. Formaldehyde, hydrogen peroxide and Ekarox B 10 were added through the system normally used for formaldehyde (16). Busan 881 and Antiformin DMT were added through an additional system. Formaldehyde, hydrogen peroxide and Ekarox B 10 were used as shock chemicals (i.e. they were added in large amounts during a short time). Busan 881 and Antiformin DMT were added either continuously or periodically. A periodical addition could be an addition for two hours followed by a pause for six hours and then a new addition for two hours. In Table 4 the different systems of disinfecting are described.

Criteria for the addition of shock chemicals

The shock chemicals formaldehyde, hydrogen peroxide and Ekarox B 10 were added into the extraction system when the microbial activity indicated a need of addition. As the pH was measured continuously in the extraction system it was used as the parameter to indicate microbial activity. When the pH decreased below a value of 5.8 in the extraction trough or 5.5 in the press water, the shock chemicals were added.

Determination of the number of colony forming units (CFU) and analysis of microbial metabolites.

The methods described earlier were used to determine the number of CFU (16). The microbial metabolites were analysed by gas chromatography and for L-lactic acid enzymatic analysis was used too (16).

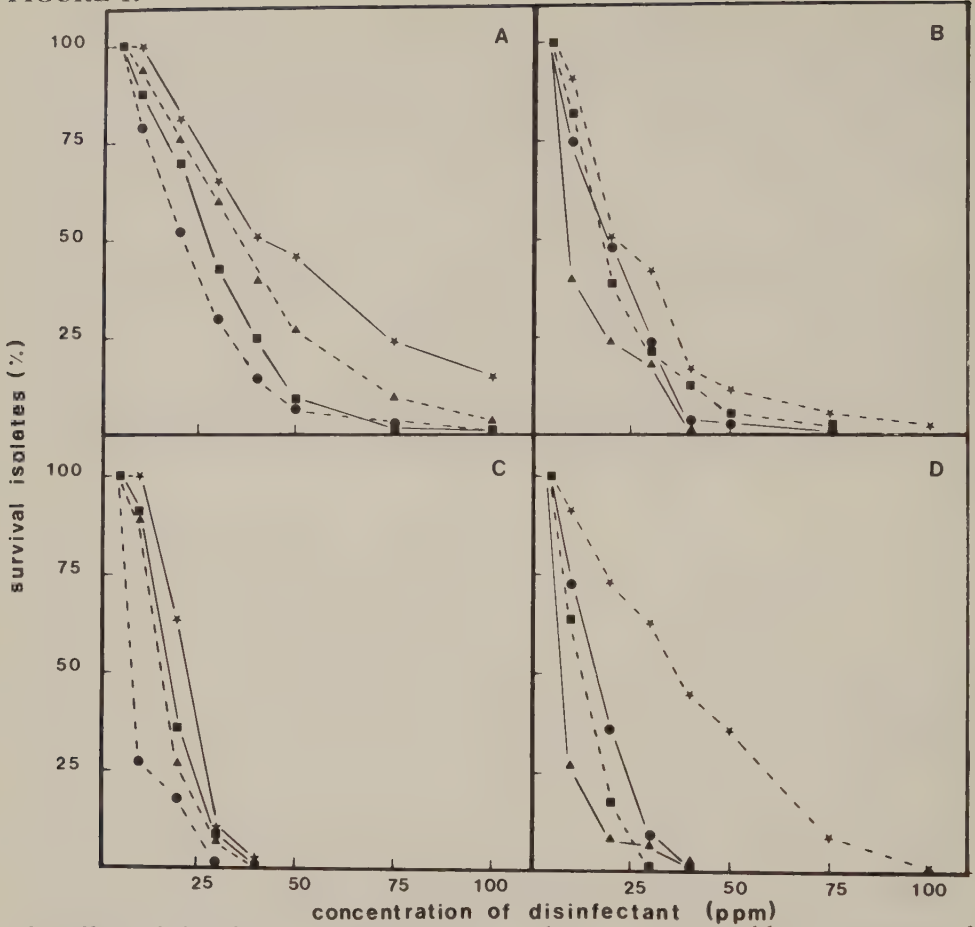
Results

Laboratory experiments

The growth inhibitory effect of the eight disinfectants measured with the agar test method is shown in Table 2. In regards to the inhibition of a broad spectrum of thermophilic bacteria the three dithiocarbamates, Ekarox B 5 and Ekarox B 10 are more effective than formaldehyde. The group of Gram positive cocci is more sensitive to all the hydrogen peroxide based disinfectants than to formaldehyde. The Gram positive spore forming bacteria, which constitute about half of the organisms tested, seem to be more resistant than the other bacteria to all the disinfectants tested. However, according to the results obtained earlier, they are not very important in the beet sugar extraction systems in Sweden. Instead bacteria of the groups Gram positive cocci and Gram positive non spore forming rods dominate. The former when formaldehyde was used and the latter when hydrogen peroxide and Ekarox B 10 were used as disinfectants. In Figure 1 the effect of concentration of the different disinfectants against bacteria belonging to these two groups is shown. A detailed investigation of the inhibitory effect of the disinfectants against the two most important groups of bacteria earlier mentioned was performed using survival experiments to determine the D values. One strain of a Gram positive non spore forming rod, strain 771, which has been identified as a *Lactobacillus* sp and one Gram positive coccus, strain 657, for which a new genus has been suggested, *Saccharococcus*, were used. The D values for these organisms are given in Table 3.

The results obtained in the two laboratory methods were in agreement with each other.

FIGURE 1.



The effect of disinfectant concentrations on the two most troublesome groups of bacteria.

A and B: Gram positive non-spore forming rods. 67 strains.

C and D: Gram positive cocci. 11 strains.

The eight disinfectants are:

Hydrogen peroxide ★ ——— ★

Ekarox B5 ■ ——— ■

Formaldehyde ★ --- ★

Antiformin DMT ■ --- ■

Ekarox B1 ▲ --- ▲

Ekarox B10 ● --- ●

Busan 881 ▲ ——— ▲

Nalco 247 ● ——— ●

TABLE 2. Amount of active substances (ppm) of the disinfectants needed for a growth inhibition of 50% of the bacterial strains tested (MIC_{50}). The figures refers to median values.

DISINFECTANT	All tested strains n = 220	Gram positive spore forming rods n = 106	Gram positive non spore forming rods n = 67	Gram negative rods n = 30	Gram positive cocci n = 11	Actino- mycetes n = 6
Formaldehyde	30	34	20	23	37	20
Hydrogen peroxide	54	66	41	32	23	26
Ekarox B1	31	37	35	33	17	30
Ekarox B5	25	27	27	28	16	20
Ekarox B10	17	20	20	16	9	20
Busan 881	12	14	9	15	8	17
Antiformin DMT	22	29	17	26	13	30
Nalco 247	18	19	19	23	16	20

TABLE 3. *D values (minutes) for a Gram positive non-spore forming rod, strain 771, and a Gram positive coccus, strain 657, at 70°C.*

DISINFECTANT	CONCENTRATION active substances (ppm)	D (minutes)	
		Strain 771	Strain 657
Formaldehyde	50	14	32
Formaldehyde	100	4,5	20
Hydrogen peroxide	50	>500	85
Hydrogen peroxide	100	50	17
Ekarox B1	50	280	60
Ekarox B1	100	55	8
Ekarox B5	30	42	7,5
Ekarox B10	20	15	4,5
Antiformin DMT	10	25	18,5
Nalco 247	10	35	38
Busan 881	10	4	3

The death rate curves were linear over 3 logarithms.

Full scale experiments

The different combinations of disinfectants tested in the sugar factories are shown in Table 4.

TABLE 4. *Different systems of disinfectants used in the full scale experiments.*

L-lactic acid and CFU: minimum values were obtained at 72°C or above and maximum values were obtained at 67°C or below.

DISINFECTANTS	Method of addition	Concentration ppm	Dominating species	pH average	L-lactic acid mg/l 2 hours after addition			CFU/ml 70°C 2 hours after addition	
					min	average	max	min	max
1. Formaldehyde	shock	200 -500	G+ coccus	6.2	10	300	1100	1×10 ¹	1×10 ⁴
2. Hydrogen peroxide	shock	200 -500	G+ rod	6.0	10	300	1200	1×10 ²	1×10 ⁶
3. Ekarox B10	shock	200 -500	G+ rod	6.1	10	300	950	1×10 ¹	5×10 ⁵
4. Busan 881	continuous	7 - 14	G+ coccus	6.0	150	300	1250	5×10 ²	5×10 ⁵
5. Busan 881	periodical	7	G+ coccus	6.3	10	150	1000	1×10 ¹	1×10 ⁴
Formaldehyde	shock	200 -500	G+ coccus	6.3	10	150	1000	1×10 ¹	1×10 ⁴
6. Busan 881	periodical	7	G+ rod	6.2	100	300	1250	1×10 ²	1×10 ⁶
Ekarox B10	shock	200 -500	G+ rod	6.2	100	300	1250	1×10 ²	1×10 ⁶
7. Antiformin DMT	continuous	8,5- 17	G+ coccus	6.0	120	200	1000	1×10 ³	1×10 ⁶
8. Antiformin DMT	periodical	8,5	G+ coccus	6.2	10	150	1000	1×10 ¹	1×10 ⁴
Formaldehyde	shock	200 -500	G+ coccus	6.2	10	150	1000	1×10 ¹	1×10 ⁴

Temperature

The temperature was found to be of great importance since it has an influence both on the activity of the microflora and the effect of the disinfectants. If the temperature was below 67°C it was very difficult to control the microflora and its activity. The optimum temperature of the two most important bacteria was in the range of 67–71°C. In this range it was also difficult to suppress their activity. At temperatures above 72°C, the microflora showed a low activity and there were few species which were able to grow.

Formaldehyde

When formaldehyde was used as the shock chemical (Table 4), the dominating organism found was the Gram positive coccus. This organism has been recorded as the dominant organism during all four years of study when formaldehyde was used as the disinfectant. The coccus was found to have the highest tolerance to formaldehyde in the laboratory test (Table 2). This efficacy of formaldehyde was less affected by changes in the temperature in the range of 65–74°C than disinfectant systems containing hydrogen peroxide (Table 4).

Hydrogen peroxide

Hydrogen peroxide was used in the same way as formaldehyde i.e. as a shock chemical (Table 4). When hydrogen peroxide replaced formaldehyde, the dominating species of the microflora changed. The Gram positive coccus, always found when formaldehyde was used, disappeared generally within 48 hours. After this time a Gram positive non-spore forming rod, identified as a *Lactobacillus* sp, began to be dominant. This organism and the coccus both had a rapid growth in the extraction system. The generation time was less than 20 minutes at 70°C. The effectiveness of hydrogen peroxide was more affected by the temperature than was the formaldehyde. At low temperatures (Table 4) the growth and activity of the microflora was greater than it was when formaldehyde was used. At high temperatures, 71°C and above, it was possible to control the microflora using approximately the same amounts of hydrogen peroxide and formaldehyde.

TABLE 5. *Characteristics of the two types of dominant species.*

CHARACTER	Gram positive coccus	Gram positive non spore forming rod
Cell form and size	Coccus Ø 1–2 µm	Rod 1×3 µm
Form aggregates	+	–
Endospore	–	–
Motility	–	–
Colony on TS agar	Yellowbrown slightly rough Ø 1–2 mm	Grey, clear smooth Ø 1–2 mm
Oxygen tolerance	Aerobic	Facultative anaerobic
Temperature minimum range °C	50–52	50–52
Temperature optimum range °C	67–70	67–71
Temperature maximum range °C	75–78	76–79
Main metabolite by sucrose degradation	L-lactic acid	L-lactic acid
Other metabolites by sucrose degradation	Acetic acid Propionic acid iso – Butyric acid	Acetic acid Ethanol

Alternating use of formaldehyde and hydrogen peroxide

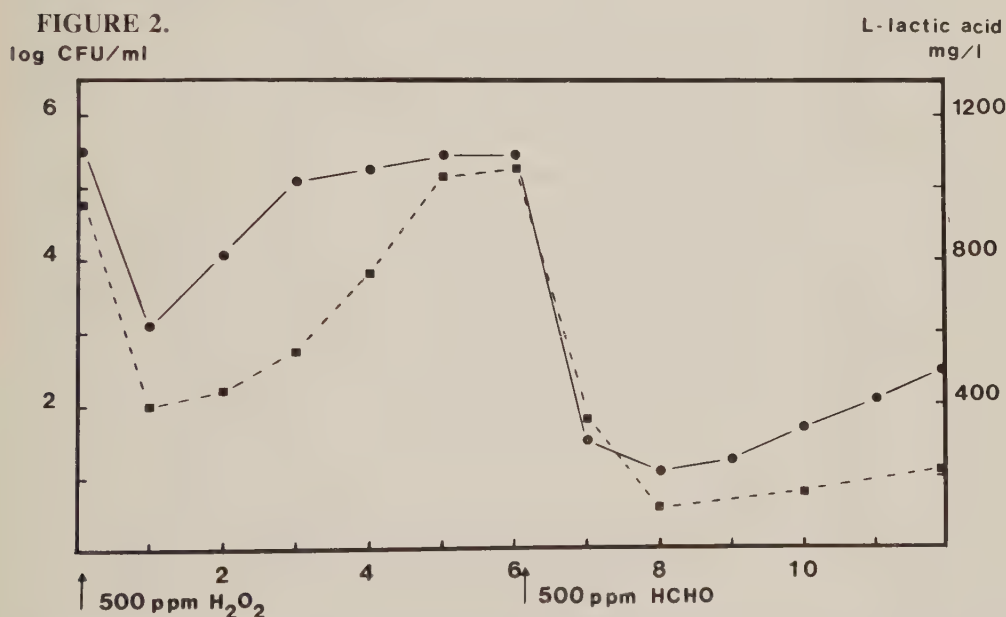
The alternating use of hydrogen peroxide and formaldehyde was advantageous. In Figure 2 the effect of changing disinfectant is shown. Formaldehyde very effectively suppressed the growth and activity of the *Lactobacillus* sp. The growth seen in Figure 2 after the addition of formaldehyde consisted of the *Lactobacillus* sp, but due to the large reduction in number its activity was very small. As the *Lactobacillus* sp after about 24 hours was no longer dominating, a new organism soon became dominant. When formaldehyde replaced hydrogen peroxide it was the Gram positive coccus that became dominant. The time before a new organism became dominant was longer at high temperatures.

It has also been observed that if the extraction system was stopped for technical reasons and emptied, the same organism became dominant after the stop as previously, if no change of disinfectant was made.

In Table 5 some characteristics are given for the two most important organisms.

Ekarox B 10

Ekarox B 10 was used a shock chemical. In the laboratory experiments, Ekarox B 10 was found to have a better antimicrobial effect than the pure hydrogen peroxide (Table 2). This was true also for the full scale experiments (Table 4). The Gram positive coccus disappeared generally within 24 hours when Ekarox B 10 replaced formaldehyde.



The effects of the addition of hydrogen (H_2O_2) 500 ppm and formaldehyde (HCHO) 500 ppm on a thermophilic *Lactobacillus* sp in beet sugar extraction. Temperature $68^\circ C$.

The additions of the disinfectants are indicated by arrows.

● ——— ● = CFU/ml at $70^\circ C$

■ - - - - ■ = lactic acid mg/l

When Ekarox B 10 was used the dominating organism was the same type of bacteria observed as when pure hydrogen peroxide was used i.e. a *Lactobacillus* sp.

The effect of temperature was the same as when hydrogen peroxide was used.

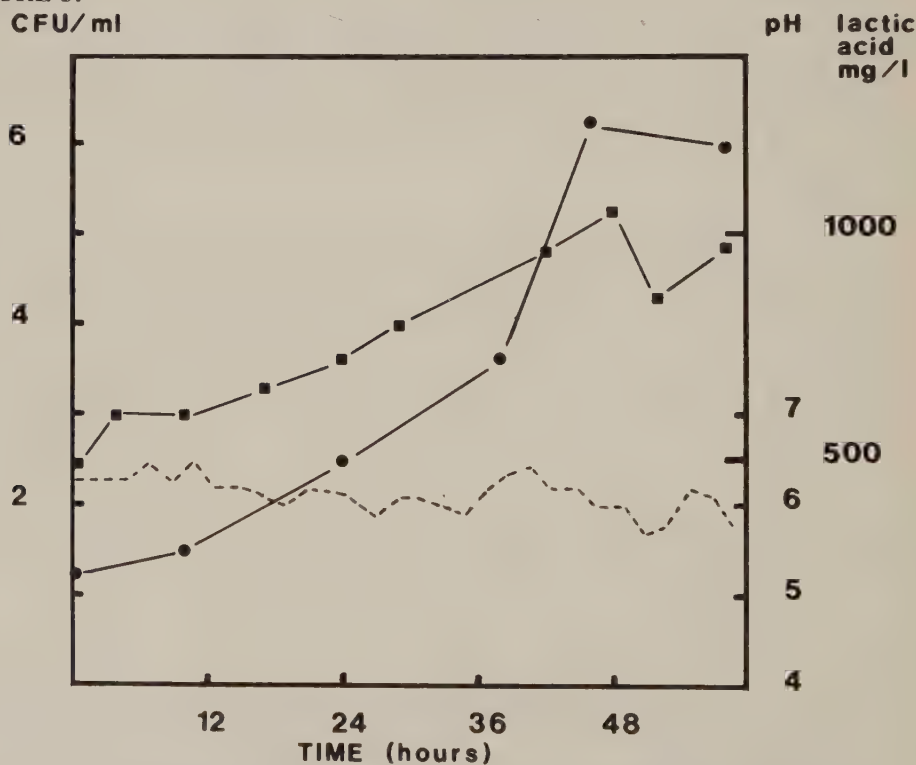
Busan 881

Busan 881 was used in two different ways. The first was a continuous addition. Two different concentrations, 7 and 14 ppm, were used. The second way was a periodical addition during two hours followed by a six hours interval and then a new addition for two hours etc. The amount of Busan 881 added this way corresponded to 7 ppm added continuously. The periodical addition allowed a temporary higher concentration of approximately 20 ppm. Busan 881 added this way was used in combination both with Ekarox B 10 and formaldehyde.

Busan 881 used alone at a concentration of 7–14 ppm added continuously or periodically could not suppress the activity and the growth of the microflora (Table 4). A period when Busan 881 was added continuously at a concentration of 14 ppm is shown in Figure 3. Neither the Gram positive coccus nor the *Lactobacillus* sp was

FIGURE 3.

log CFU/ml



Effect of a continuous addition of 14 ppm of Busan 881. The dominating micro-organism was the Gram positive coccus tentatively named *Saccharococcus thermophilus*.

Ekarox B 10 was added in a concentration of 70 ppm at 50 hours due to a drop in pH.

● ——— ● = CFU/ml at 70°C

■ ——— ■ = lactic acid mg/l

----- = pH

suppressed by Busan 881 alone at these concentrations. This means that the dominating organisms of the formaldehyde periods and the Ekarox B 10 periods were not changed by Busan 881.

When Busan 881 was used in combination with formaldehyde or Ekarox B 10 it was found to have an effect upon the amounts of shock chemicals needed (Table 6). The amounts of formaldehyde and Ekarox B 10 needed were decreased by 50 % compared to periods when no Busan 881 was used. Because the Busan 881 was added in low concentrations during a long time (compared with the shock chemicals) the effect was limited.

Busan 881 was not able to suppress and change the dominating microflora. The dominating organisms were not selected by the Busan 881 but by the shock chemical used.

TABLE 6. *The effect of Busan 881 on the amounts of formaldehyde and Ekarox B10 needed to suppress the microbial activity.*

Busan 881 Method of addition	Ekarox B10 litres/24 hours	Formaldehyde litres/24 hours
No addition	1790*	1273
7 ppm periodically	820*	642
7 ppm continuously	321	ND
14 ppm continuously	165	ND

* = Low temperature for Ekarox B10

ND = not determined

Antiformin DMT

Antiformin DMT was used by continuous addition in concentrations of 8,5 or 17 ppm. It was also, like the Busan 881, added periodically. For Antiformin DMT 3 hours addition followed by a pause of 5 hours, etc. was used. The addition corresponded to 8,5 ppm added continuously. By this method of addition the Antiformin DMT was supported by formaldehyde as a shock chemical (Table 4).

The Gram positive coccus was the dominant species in the extraction when the addition of Antiformin DMT began because the disinfectant used previously was formaldehyde. The coccus was not suppressed at these concentrations (Table 4) by the Antiformin DMT alone, regardless of the manner for Antiformin DMT addition. In fact, the Antiformin DMT was found to be less effective than Busan 881. This was also in accordance with the laboratory results.

Like Busan 881 Antiformin DMT was added in low concentrations and, therefore, was not able to suppress and change the dominating micro-organism.

However, when the Antiformin DMT was supported by formaldehyde as a shock chemical the result was nearly as effective as for the combination of Busan 881 and formaldehyde (Table 4).

Discussion

The large number of thermophilic bacteria isolated from the beet sugar extraction were very useful in laboratory investigations of the disinfectants. The actual extraction environment is very complex which makes exact laboratory simulation impossible. One alternative then is to work with isolates taken from the system under study.

The disinfectant is one of the selecting environmental parameters which is important in order to determine which organism should be dominant in the extraction system (16).

The Gram positive cocci and the Gram positive spore forming bacteria showed the highest tolerance to formaldehyde. Of these organisms some strains of the Gram positive cocci were capable of short generation time in the extraction system and, therefore, could become dominant. These cocci were demonstrated to be sensitive to other disinfectants (22).

The intension of the laboratory investigation was to compare the relative toxicities of the disinfectants compared to that of formaldehyde. It must be noted that the figures of inhibiting concentrations are only valid for the laboratory conditions used. However, laboratory investigations are useful in order to gain a knowledge of the relative effectiveness of different disinfectants. In this investigation, the results obtained for the most important organisms in the laboratory investigation were in full agreement with the results later found in the extraction system.

Matteuzzi et al (19) investigated the sensitivity of *Bacillus stearothermophilus* and found that Busan 881 and Nalco 247 were more toxic than formaldehyde, while the effect upon *Clostridium thermohydrosulfuricum* was less than that of formaldehyde. The sensitivity of a *Bacillus* sp to Nalco 247, Antiformin DMT, Busan 881, hydrogen peroxide and formaldehyde was determined by Nickisch-Hartfiel and Mauch (21). The relative toxic effect of these substances to the *Bacillus* sp were similar to those found in this investigation.

In the full scale experiments the temperature was found to be of great importance. Generally it was more difficult to suppress the growth and activity of the microflora when the temperature decreased from 70–72°C to 67°C or below. This was probably caused by the fact that at lower temperature a greater number of different bacterial species can grow including the most troublesome bacteria which still have a considerable activity (16). Temperature also influences the activity of different disinfectants. Generally the activity increases with increasing temperature. This has been reported for formaldehyde (32) and hydrogen peroxide (25, 31). It was also noted in the product information for Busan 881.

Nickisch-Hartfiel and Mauch (21) observed that the amounts of formaldehyde needed increased at the end of the campaigns. They suggested that this phenomena was due to adaptation and development of resistance to formaldehyde. During four years of study in seven Swedish factories there was no tendency found for micro-organisms to become more resistant at the end of the campaigns. The same amount of formaldehyde was required to suppress the Gram positive coccus at the end of the campaigns as at the beginning and in the middle. Formaldehyde is used as a shock chemical. This fact reduces the risk that a micro-organism can develop a resistance compared to addition of the disinfectant continuously.

If the temperature must be decreased for technical reasons (e.g. lack of steam, frozen and thawed beets), the amounts of disinfectant needed will increase.

Although hydrogen peroxide and Ekarox B 10 had a good inhibitory effect, the results obtained in full scale experiments showed that they were very sensitive to low temperatures. Other workers (35) reported that the amount of hydrogen peroxide needed was about half that of formaldehyde, but this was not true for this study. In the best case the amount of hydrogen peroxide required was equal to that of formaldehyde.

The usefulness of dithiocarbamates in beet sugar extraction is limited. First it must be noted that the substances can not be added in large amounts due to recommendations of their use (9). This gives the second problem: in low concentrations, the dithiocarbamates are unable to suppress or change the dominating microorganisms in the temperature range of 64–72°C.

Continuous addition is not recommended since the risk of adaptation of the microflora will increase (21). The ideal way to add these substances is periodically. When the periodical system was combined with a shock chemical, the amounts of shock chemical required was reduced. When Busan 881 alone was added as a shock chemical, almost no effect at all was achieved.

The alternating use of formaldehyde and Ekarox B 10 gave a good result. This was due to the fact that there was only one dominant species present for each of these disinfectants and there was a more effective suppression of the organism tolerant to formaldehyde with Ekarox B 10 and *vice versa*. The use of several alternating disinfectants has also been proposed by other authors (19, 21).

The aim of the full scale experiments, to find an alternative to formaldehyde, was fulfilled. The system with Ekarox B 10 as shock chemical can be an alternative to formaldehyde, contingent on having a temperature of at least 70–72°C. Also the system with Ekarox B 10 as shock chemical and Busan 881 periodically added is possible to use with the same criteria for temperature as for Ekarox B 10 alone.

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